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Exposure to extracts from Porphyromonas gingivalis alters gene expression of osteoblasts in vitro

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Exposure to Extracts from Porphyromonas gingivalis Alters Gene Expression of Osteoblasts In vitro

by

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THESIS

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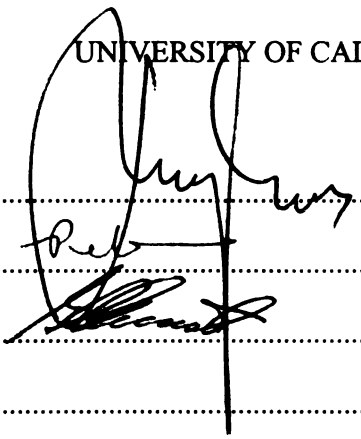

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Dedications

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Abstract:

Periodontopathogenic microorganisms such as *Porphyromonas gingivalis* are considered important etiologic agents in the initiation and progression of periodontitis. Virulence factors such as the lipopolysaccharides of outer membranes of *Porphyromas gingivalis* have been associated with the inflammatory destruction of periodontal tissues. *P. gingivalis* induces the release of cytokines and inflammatory mediators from a variety of cells including macrophages and fibroblasts. The ensuing inflammatory response can cause local destruction of periodontal tissues. However, the direct effects of extracts from *P. gingivalis* on osteoblast gene expression have not been fully elucidated. In this *in vitro* study, the effects of these extracts on gene expression of a human fetal osteoblast cell line (hFOB 1.19) were investigated. *P. gingivalis* was grown in culture and extracts were prepared by sonication of whole bacterial cells. Lysates were centrifuged to separate the insoluble fraction. The resulting soluble supernatant was freeze-dried and added to hFOB cell culture medium for final concentrations of 1, 10 and 100μg/ml. Cells were cultured for 4 days. The expression profile of osteogenesis-related genes of hFOB 1.19 cells, including those involved in extracellular matrix production, cytokines, growth factors, internal signaling molecules and cell adhesion were determined using gene microarrays. The results revealed that extracts from *P. gingivalis* profoundly reduced (54.6% to 98.9%) mRNA expression of proteins associated with collagen production (collagen type II, III and XI). Furthermore, signaling proteins such as BMP-2, BMP-3, TGF-2, TGF-3, IGF-1, and IGF-2 were also downregulated with a range of 17.4% to 157.6% reduction. Genes of various internal signaling molecules and external receptors related to cytokines and growth factors were also downregulated, but to a lesser extent. Markers of osteoblast differentiation such as osteocalcin and osteopontin were also reduced (range 4.3% to 98%) in response to extracts from *P.*

gingivalis. However, alkaline phosphatase expression (ALP) mRNA expression, a known marker of osteoblast differentiation and maturation was increased to a maximum 22 fold in response to various concentrations of extracts from *P. gingivalis*. Overall, these results indicate that soluble extracts from *P. gingivalis* inhibit the expression of genes important in early formation of bone, including matrix production, growth factors, and internal signaling molecules of hFOB cells *in vitro*.

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(I) Background and Significance:

(i) Introduction:

Periodontal diseases are multifactorial infections elicited by a complex of bacterial species that interact with host tissues and cells. A chronic local polymicrobial challenge to periodontal tissue triggers an intricate host response composed of a broad array of inflammatory mediators, cytokines, chemokines, and other products derived from host cells in the gingival tissues as well as emigrating inflammatory cells. This local tissue response can lead to host tissue destruction, the hallmark characteristic of periodontal disease. Loss of tooth supporting tissues such as the periodontal ligament, alveolar bone, and connective tissue attachment can lead to clinical findings such as deep periodontal pockets, periodontal inflammation, bleeding on probing, tooth mobility, recession, and eventual tooth loss. Both the host response and bacterial challenge are necessary components for periodontitis to occur. The complex interplay between the bacterial challenge and innate and acquired host responses ultimately determines the disease outcome.

Periodontopathogenic microorganisms are considered important etiologic agents in the initiation and progression of periodontitis. These microorganisms may mediate periodontal tissue destruction either directly through their multiple virulence factors, or indirectly by host-mediated imflammatory responses. (Miguel et al., 2003)[37] Several pathways have been proposed by which bacteria and their products may cause alveolar bone loss in periodontal diseases: (i) the direct action of bacterial products on bone cells and tissue, (ii) the direct action of bacterial products on osteoprogenitor cells (i.e., promotion of differentiation into osteoclasts), (iii) bacterial stimulation of host connective tissue cells to release mediators for induction of progenitor cells into osteoclasts, (iv) bacterial stimulation of resident connective tissue cells to release products

that function in collaboration with bacterial products to stimulate bone resorption, and (v) bacterial stimulation of connective tissue cells to release products that act directly upon bone tissue. (Hausmann et al., 1974)[20] The bacteria-induced host cell products include inflammatory cytokines and mediators of inflammation. While the exact mechanisms causing bone loss in periodontal diseases are not known, it is likely they include a combination of several or all of the above pathways. Thus, furthering our understanding of the specific pathogenic mechanisms by which bacteria mediate periodontal tissue destruction is important to formulate more effective therapeutic regimens for periodontal diseases.

(ii) *Porphyromonas gingivalis*:

P. gingivalis is a well known periodontal pathogen associated with different forms of periodontal disease including aggressive and chronic periodontitis. *P. gingivalis* is a gram-negative, anaerobic, non-motile short rod. This species has been shown to be present in increased numbers and detected more frequently in deteriorating periodontal sites in humans as well in experimentally induced periodontal lesions in dogs, sheep, and monkeys. (Haffajee and Socransky et al., 1994)[18] Socransky and co-workers (1998)[50] catalogued and stratified the oral microbiota from 185 human patients into groups or complexes representing bacterial consortia that appears to occur together and that are associated with the development of subgingival biofilms. Six closely associated groups of bacterial species were recognized. The bacteria associated with early colonization of the tooth surface and proliferate at an early stage included the yellow, green, and purple complexes. The growth of these early colonizers usually preceded the multiplication of the gram-negative orange and red complexes. The orange complex bacteria increase as plaque matures and are thought to bridge the early colonizers and the red complex

species. *P. gingivalis*, *Tannerella forsythia,* and *Treponema denticola* are the members of the red complex. Red complex species have the strongest association with clinical parameters such as probing depth, sites with gingival inflammation, and bleeding on probing. (Socransky et al., 1992)[49] (Holt et al., 2005)[24] (Amano et al., 2003)[1] It was found that a difference between a healthy and diseased site was the increased prevalence and counts of the red complex bacteria in subjects with periodontal disease. Although the red complex bacteria have the highest association with periodontal destruction, it has also been detected in non-progressing lesions. Its presence was not a prerequisite for periodontal destruction. (Socransky et al., 1998)[50]

(iii) Virulence factors of *Porphyromonas gingivalis*:

Many gram-negative anaerobic species belonging to the genera *Bacteriodes, Prevotella*, and *Porphyromonas* possess a host of virulence factors and proinflammatory components that make these bacteria common in both systemic and oral infections. (Duerden et al., 1994)[16] *P. gingivalis* produces a variety of intrinsic and extrinsic substances that are implicated in periodontal destruction. Such factors include a variety of collagenases, proteases, hemolysins, and endotoxins. (Botta et al., 1994)[8] Some products of *P. gingivalis* have been shown to inhibit collagen synthesis. (Meghji et al., 1992)[36] In addition, certain strains of *P. gingivalis* have been shown to be capable of invading epithelial cells in vitro. (Duncan et al., 1993)[17] Established virulence factors of *P. gingivalis* are listed in table 1. This commensal, opportunistic pathogen has evolved numerous mechanisms and strategies to effectively compete in the complex subgingival environment. *P. gingivalis* demonstrates multiple components that alter host cell and tissue function, and decrease or even negate host protective responses. This combination of

adaptive capabilities make this microorganism a significant component of the climax biofilm linked to periodontal tissue destruction.

(iv) Lipopolysaccharide:

A major pathogenic component of *P. gingivalis* is lipopolysaccharide (LPS; endotoxin) that is a component of its outer membrane. The outer membrane is impermeable to large molecules and hydrophobic compounds from the environment. Endotoxin (LPS) is located on the outer surface of the membrane, where it mediates contact with the environment. LPS is essential to the function of the outer membrane, and as a structural component of the cell. Lipopolysaccharides are complex amphiphilic molecules with a molecular weight of about 10kDa, that vary widely in chemical composition both between and among bacterial species. The general architecture of LPS is shown in diagram 1. LPS consists of three components or regions, Lipid A (region I), an R polysaccharide (region II) and an O polysaccharide (region III). The lipid A region is the lipid component of LPS. It contains the hydrophobic, membrane-anchoring region of LPS. Lipid A consists of a phosphorylated N-acetylglucosamine (NAG) dimer with 6 or 7 fatty acids (FA) attached. (Rietschel et al., 1987)[45] The structure of Lipid A is highly conserved among gram-negative bacteria. (Dixon et al., 2005)[14] Region II is comprised of the R core polysaccharide and is attached to the 6 position of one NAG from the lipid A portion. The R polysaccharide consists of a short chain of sugars. Two unusual sugars are usually present in the core polysaccharide for most of the gram-negative bacteria, heptose and 2-keto-3-deoxyoctonoic acid (KDO). The last region (III) or the O polysaccharide is attached to the core polysaccharide. It consists of repeating oligosaccharide subunits made up of 3-5 sugars. The individual chains vary in length ranging up to 40 repeat units. (Caroff et al., 2003)[13] The O polysaccharide is much

longer than the core polysaccharide, and it maintains the hydrophilic domain of the LPS molecule. Great variation occurs in the composition of the sugars in the O side chain between species and even strains of gram-negative bacteria. (Paramonov et al., 2001)[42] At least 20 different sugars are known to occur and many of these sugars are characteristically unique dideoxyhexoses, which occur in nature only in gram-negative cell walls. The biological effects of LPS are summarized in table 2.

The LPS from *P. gingivalis* does not have the configuration that is typical of other gram-negative bacteria in that it lacks heptose and 2-keto-3-deoxyoctonate in the R polysaccharide region. It is believed that this change in the LPS configuration induces the host to produce high amounts of anti-lipopolysaccharide IgG1, IgG2 and IgG3 antibodies. (Schenck et al., 1987)[46] LPS has multiple inflammatory consequences in the host and has been implicated to play a role in the destruction of periodontal tissues. For example, LPS extracted from *P. gingivalis* has been shown to stimulate the differentiation of osteoclastic cells by mediating inflammatory cytokines such as interleukin-1 [IL-1], (Bom-Van Noorloos et al., 1990)[5] tumor necrosis factor-alpha [TNF-α], (Reddi et al., 1996)[44] and prostaglandin E_2 [PGE_2]. (Hausmann et al., 1972)[19] LPS has also been known to trigger the release of inflammatory osteolytic factors from osteoblastic cells and to stimulate alveolar bone resorbtion. These LPS-stimulated actions are mediated by binding LPS and CD14 surface molecules, which are predominantly on monocytes/macrophages and neutrophils. (Amano et al., 1997, 2003)[1,2] (Pugin et al., 1993)[43] Multiple studies have demonstrated that the LPS from *P. gingivalis* have significant suppressive effects on periodontal fibroblasts (Marquez-San Miguel et al., 2003)[34] (Steffen et al., 2000)[52], macrophages (Bodet et al., 2005)[4] and osteoblasts. (Loomer et al., 1994, 1998)[32,33]

(v) Host Reponse to Periodontopathogenic Bacteria:

The association between the community of bacterial species that colonize gingival crevices and the onset and progression of periodontal diseases has been well established. (Zambon et al., 1990)[59] (Socransky and Haffajee, 1991, 1992)[48,49] The complex mechanisms by which bacteria in subgingival biofilms cause connective tissue destruction are only partially understood. One of the major sequellae of periodontal disease is bone loss, which results from the stimulation of bone resorption and the inhibition of bone formation when normal bone metabolism becomes uncoupled in disease. (Hausman et al., 1974)[20] Bacteria that populate the periodontal pocket, release LPS and other bacterial products into the sulcus, affecting both inflammatory and host cells in the connective tissue and osteoblasts in the adjacent bone. The periodontium is comprised of numerous cell types, including fibroblasts, epithelial cells, and an inflammatory infiltrate of polymorphonuclear cells and mononuclear cells, all of which may contribute to the inflammatory process. Monocytes are thought to be key players in the release of inflammatory cytokines. However a growing body of evidence suggests that fibroblasts and epithelial cells that were previously thought to primarily function as physical barriers and structural components of the periodontium are now recognized to play important roles in the secretion of molecules that contribute to the inflammatory process. (Bodet et al., 2005)[4] (Duncan et al., 1993)[17] (Hill et al., 1996)[21] (Marquez-San Miguel et al., 2003)[34] (Miguel et al., 2003)[37] (Steffen et al., 2000)[52] These molecules include IL-1α, IL-1β, IL-16, PGE_2 and TNF-α. In short, these compounds increase osteoclast formation and activation as well as inhibit osteoblast function, resulting in a net loss of bone.

(vi) *In Vitro* Osteoblast Models:

Of the *in vitro* osteoblast model systems that have been established to characterize osteoblast growth and differentiation, the two that are the most widely used are primary cultures obtained from human bone fragments and osteosarcoma cells derived from human bone tumors. (Subramanian et al., 2002)[53] Primary osteoblast cultures are excellent systems for study, but are limited by a heterogeneous cell population, slow growth rate, and terminal differentiation. Therefore, immortalized osteosarcoma cell lines are popular for studying osteoblast behavior because they tend to be more homogeneous and have a longer life span. However, osteosarcoma cells such as the MG-63 human osteosarcoma cell line, do have distinct disadvantages: they do not exhibit the complete phenotype of differentiated osteoblasts, have abnormal growth properties including loss of cell contact inhibition and responses to hormones and cytokines, and have obvious chromosomal defects. (Spelsberg et al., 1995)[51] To overcome these limitations, Spelsberg and co-workers (1995)[51] established a human fetal osteoblast cell (hFOB 1.19) by transfecting fetal bone-derived osteoblast cells with a temperature-sensitive mutant of the SV40 T antigen. This cell line exhibits properties more akin to normal osteoblasts such as expression of osteoblastic markers, response to cytokines and hormones, fewer chromosomal abnormalities, and the ability to synthesize bone matrix similar to differentiated osteoblasts. In addition, these cells have the ability to form mineralized nodules *in vitro*. The hFOB cell line has been increasing in popularity due to both its advantages and the disadvantages observed in the osteosarcoma lines such as the MG-63.

(vii) Gene Microarray:

The advancement of nucleic acid microarray technology has made it possible to analyze the expression of multiple genes in a single experiment. Commercial products of gene microarrays allow for a quick and economical method of detecting specific gene expression. Specifically, these experiments are concerned with the gene expression of human osteoblasts. The GEArray Q series Human Osteogenesis Gene microarray included genes that regulate osteogenesis including growth factors such as bone morphogenetic protein (BMP), transforming growth factor-beta (TGF-β), extracellular matrix proteins, (collagen) and their internal signaling molecule (Smad) as well as some skeletal-related genes known to play important roles for bone development. The GEArray Q series contain gene specific cDNA fragments that are printed on a 3.8 x 4.8 cm nylon membrane with a 8 x 14 grid. This system has the advantage over many other methods of gene expression profiling in that the expression of a large number of known genes (up to 96) relevant to the biological process being studied can be assessed in one hybridization experiment. Each cDNA fragment is printed with a tetra-spot format for easy pattern identification. Although, the GEArray consists of many genes related to osteogenesis, it is by no means an exhaustive collection of all the genes involved and cannot detect novel or uncharacterized gene expression.

(viii) Previous Studies:

Previous studies have shown that extracts and metabolic products derived from *P. gingivalis* 2561 significantly inhibit bone formation and induce resorption of dentine slices and calcium-phosphate thin films (bone-like tissue). (Loomer et al., 1994, 1995, 1998)[30,32,33]

Whole sonicated extracts from *P. gingivalis* and other periodontopathogenic bacteria (including *Actinobacillus actinomycetemcomitans, Prevotella intermedia* and *Treponema denticola)* were shown to inhibit bone formation in a dose-dependent manner. Varying amounts of inhibitory activity were observed, depending on the molecular size range of the extract, with very profound inhibitory effects observed in the < 5 kDa range. Further characterization of the *P. gingivalis* extracts revealed that both proteinaceous and non-proteinaceous products, including lipopolysaccharide, were able to inhibit osteogenesis. Similar findings were obtained using chick and rat bone marrow stromal cell cultures and human MG-63 cell line. (Loomer et al., 2002)[31] The data suggest that another mechanism through which periodontopathogens such as *P. gingivalis* might possibly contribute to the bone loss in periodontal diseases is by inhibiting bone formation directly.

Meghji and co-workers (1992)[36] showed that readily soluble surface-associated material (SAM) isolated from *Actinobacillus actinomycetemcomitans*, *P. gingivalis* and *Eikenella corrodens* was able to inhibit DNA and collagen synthesis in murine calvaria tissue cultures at concentrations as low as 10 μg/ml. The most active inhibition came from the SAM from *P. gingivalis* and *Eikenella corrodens*. These experiments demonstrated that low concentrations of products from the surface of periodontopathic bacteria are able to induce a response that may play a role in the destruction of periodontal tissues. Kadono and co-workers (1999)[27] also used rat calvariae tissues, but specifically focused the study on the effects of LPS extracted from *P. gingivalis*. It was found that LPS from *P. gingivalis* significantly inhibited bone nodule formation by up to 27% of the control value and decreased alkaline phosphatase (ALP) activity to 60% compared to control levels. The expression of mRNA for two matrix proteins related to bone metabolism, osteocalcin and osteopontin, were markedly suppressed by LPS from *P.*

gingivalis. Furthermore, the addition of the extracts increased the expression of mRNA for CD14, LPS receptor and IL-1B. These results indicated that LPS has a direct effect on bone tissue cultures and suggests that this effect may play a role in the bone resorption in periodontal disease.

In addition to the direct effects of extracts from *P. gingivalis* and other periodontopathogens on osteoblast cells, bacterial extracts have been shown to have a direct effect on other cells of the periodontium. Previous studies of gingival fibroblasts have shown that their phenotype and function are altered at sites with periodontal disease. Marquez-San Miguel and co-workers (2003)[34] observed a significant suppressing effect of extracts from *P. gingivalis*, *Tannerella forsythia* (*Bacteroides forsythus)*, and *Treponema denticola* on the proliferation rate of fibroblasts extracted from the periodontal ligament of human 3rd molar roots. A 45% decrease in the expression of ALP was seen after with the addition of the bacterial extracts. This effect was seen within 24 hours of application of the bacterial extract. Hill and Ebersole (1996)[21] observed human fibroblast tissue cultures with the addition of LPS from *Actinobacillus actinomycetemcomitans*. LPS at high concentrations (> 9μg/well) resulted in a reduction in DNA synthesis by gingival fibroblasts. However, lower concentrations of LPS (< 9μg/well) showed a minimal enhancement of DNA synthesis. Furthermore, fibroblasts that were primed with high doses of LPS significantly inhibited mitogenesis when known growth factors such as insulin, epidermal growth factor, platelet-derived growth factor, and transforming growth factor were subsequently added. Results showed that the longer the high doses of LPS were preincubated with the cells, the greater the inhibitory effect. Steffen and co-workers (2000)[52] further demonstrated that *P. gingivalis* significantly affected the induction of proinflammatory mediators

in human tissue fibroblast cultures. Both viable and killed *P. gingivalis* cells were capable of inducing cytokine production from fibroblasts, such as prostaglandin-E_2, IL-1β, IL-6 and IL-8.

Previous studies have also shown that *P. gingivalis* directly affects epithelial cells and monocytes in the periodontium. Bodet and co-workers (2005)[4] investigated the effects of the red complex bacteria on co-cultures of monocytes and epithelium in vitro. Following stimulation of the culture with whole cells of *P. gingivalis*, there was induced production of IL-β, IL-6, IL-8, PGE_2 and matrix metalloproteinase-9 (MMP-9) in comparison to non-bacterial challenged cultures. However, LPS extracts from *P. gingivalis* were only able to stimulate the production of IL-8, MMP-9 and PGE_2. This study shows that *P. gingivalis* may induce high levels of proinflammatory mediators and MMP-9 in periodontal lesions, which could contribute to periodontal disease progression.

(II) Specific Aim and Hypothesis

The aim of this research was to investigate the effects of periodontopathogenic bacteria on gene expression of osteoblastic cells. Specifically, we examined the effects of sonicated extracts and isolated products, such as lipopolysaccharide, from *Porphyromonas gingivalis* 2561 on the gene expression of a well-characterized human osteoblastic cell line hFOB 1.19 (Human fetal osteoblasts). We utilized a cDNA expression microarrays that contain cDNA fragments from genes associated with osteogenesis.

The null hypothesis was that the sonicated extracts and isolated products from *P. gingivalis* will not alter the expression of genes involved in the process of osteogenesis. The alternate hypothesis was that sonicated extracts and isolates from *P. gingivalis* will alter the expression of genes involved in the process of osteogenesis. The genes evaluated are those involved in the production or regulation of growth factors (such as BMPs and TGF-ß), internal cell signaling (the Smads), and osteoblast matrix and associated protein production (e.g., type I collagen, osteocalcin, MMPs, osteopontin). These results should help clarify our understanding of the pathogenic mechanisms in periodontal disease, which could ultimately lead to improved, more targeted and cost-effective therapies. They will also have direct relevance to surgical periodontal regeneration, where bone formation is the dominant ongoing biological process.

(III) Materials and Methods

(i) Bacterial Culture Conditions:

Porphyromonas gingivalis strain 2561 (ATCC 33277) was grown on blood agar plates at 37°C in an anaerobic chamber in an atmosphere containing 80% N_2, 10% H_2, and 10% CO_2. Blood agar plates were made from 465 ml distilled water, 25 ml laked rabbit blood, 21.5g Brucella Agar Base (Difco), 2.5 ml of 50mg/ml hemin and 0.5 ml of 1% dithiothreitol. After 5 to 7 days growth, bacterial colonies were scraped from the agar plates and stored at –20° C. A total of 9 grams of bacteria were harvested.

(ii)Bacterial Extract Preparation:

Bacterial cells were harvested by centrifugation at 10,000 x g for 15 minutes at 4°C. The bacterial cell pellet was resuspended in 50 ml of phosphate-buffered saline, pH 7. The bacterial suspension was then sonicated at maximum power output using a Biosonik IV sonicator (Bronwill Co., Rochester, NY). Complete sonication of the bacteria was verified by light microscopy. The insoluble debris was then be removed by centrifugation at 10,000 x g for 30 minutes at 4°C. The soluble fraction in the solution was separated from the insoluble pellet and freeze-dried to a powder-like consistency.

The freeze-dried extracts from *P. gingivalis* were then resuspended in α-MEM (minimum essential medium) solution and sterilized by filtration using a 0.45 μm pore size filter (Millipore®). The α-MEM containing the *P. gingivalis* extracts were used to create a supplemented cell medium (see below) with a final concentration of extracts of 100μg/ml, 10μg/ml and 1 μg/ml.

(iii) Osteogenic Cell Cuture Conditions:

For this study, the human osteoblastic cell line hFOB 1.19 was used. The hFOB 1.19 cells were obtained from the ATCC (American Tissue Culture Collection). Cells from frozen stock were incubated for 5 days, at 37°C in a humidified atmosphere containing 5% CO_2, in α-MEM supplemented with 15% fetal calf serum, ascorbic acid, dexamethasone and glycerophosphate (See below for supplemented media). The cells were subsequently subcultured onto plates for use in the experiments.

(iv) Experimental Culture Conditions:

Cultures of hFOB cells were incubated with supplemented media containing *P. gingivalis* extracts at concentrations of 100μg/ml, 10 μg/ml and 1 μg/ml. Parallel cultures of hFOB cells inoculated with regular supplemented media served as controls for comparison of gene expression. Cells were cultured in 3ml 6-well culture dishes for 4 days to reach confluence. Confluence was verified microscopically using a phase contrast microscope. Supplemented media consisted of the following:

500 ml α-MEM (miminum essential medium)
75 ml Fetal Bovine Serum
5 ml Penicillin Streptomycin 1%
5 ml 10^{-2} Fungizone
5 ml 10^{-2} B-glycerophosphate
4 ml Ascorbic Acid (15ug/ml)
50μl Dexamethasone 10^{-8M}

A separate 6-well culture dish was incubated with supplemented media with the following *P. gingivalis* extract concentrations: 0 (none; controls), 1μg/ml, 10 μg/ml, and 100μg/ml. Culture plates were then incubated in a chamber at 37°C in a humidified atmosphere containing 5% CO_2 for an additional 4 days. Supplemented media with its respective concentration *P. gingivalis* extracts were replaced every 48 hours. After 4 days, the cells were removed from the incubator, the supplemented media was aspirated from the dish and washed several times with phosphate buffer (pH 7.2). Plates were then stored at –80° C for further analysis.

(v) Gene Expression Methodology:

To profile the osteogenesis and inflammatory cytokine gene expression of hFOB 1.19 cells, the system developed by SuperArray Inc. (Bethesda, MD) was utilized. Specifically, the GEArray Q series was designed to profile gene expression in the process of osteogenic differentiation. The overall process is shown in Diagram 2.

(a) RNA Isolation: Total RNA was isolated using RNeasy mini kits (Qiagen Inc., Valencia, CA). Briefly, this procedure consists of the following steps: (a) Cells grown in a monolayer were directly lysed in the tissue culture plate using the supplied buffer containing beta mercaptoethanol, (b) The lysates were then homogenized by passing the contents 5 times through a 10-gauge needle (c) Total RNA was isolated by passing the lysates through a RNeasy columns with different buffers to elute the final RNA.

RNA isolates were obtained from each of the cell cultures at day 0 and at day 4 for each of the experimental conditions: control, 1μg/ml, 10 μg/ml, and 100μg/ml.

The quality of the RNA was assessed by spectrophotometry. To ensure that good quality RNA was obtained, the ratio of absorbance reading at 260nm / 280nm was assessed. Good quality RNA that has not degraded and does not contain other impurities such as proteins and free nucleotides should have an absorbance reading higher than 1.8. The spectrophotometry readings at 260nm/280nm for the RNA isolates in these experiments were within the range of 1.90 to 2.10. The RNA isolates in these experiments were of average RNA content, yielded concentrations ranging from 625mg/ml to 2.1g/ml of RNA per isolate. A total of 10 µg of RNA was necessary for probe synthesis. After isolation of the RNA, samples were stored in a –80° C freezer. For the remainder of the steps, the GEArray kit (SuperArray Inc.) was used. A majority of the needed reagents can be found in these kits, although some require separate purchase (such as 32P-dCTP, reverse transcriptase, RNAase inhibitor).

(b) cDNA Probe Synthesis: cDNA Probes were synthesized using the protocol for chemiluminescent detection. For accuracy, a PCR thermocycler was pre-programmed and used for most of the reactions. The total RNA isolated in step (i) was used as the template for the 32P-cDNA probe. A total of 10 µg of RNA was needed per probe synthesis. The annealing mix was prepared by combining the RNA and a buffer solution provided by the GEArray kit (GEA primer buffer). The combined solution was heated to 70° C for 3 minutes, cooled to 42° C and incubated for 2 minutes. The labeling mix was prepared by mixing GEA labeling buffer (provided by the kit), 32P-dCTP, RNAase inhibitor, reverse transcriptase enzyme and RNAase-free water. The annealing and the labeling mix were incubated together at 42° C for 90 minutes. The probe solution was then denatured by addition of a denaturing solution provided by the kit (buffer D) and heated to 68° C for 20 minutes. Denaturization was stopped with a neutralizing solution provided by the kit (buffer E) at 68° C for 10 minutes.

Probes were amplified in the linear polymerase reaction (LPR) step. Briefly, a LPR cocktail containing the previous step's probe, DNA polymerase, and Biotin-16-dUTP was amplified using the PCR thermocycler under the following conditions:
85° C for 5 min; 30 cycles of (85° C, 1 min, 50° C 1 min, 72° C 1 min); then 72° C for 5 min. The cDNA probes were denatured by heating the contents to 94° C for 2 min and then quickly chilling on ice. The cDNA probes were then ready for hybridization.

(c) Hybridization: GEArray Q series Human Osteogenesis Gene array (SuperArray, Frederick MD) was used for all hybridization experiments. The hybridization was carried out at 68° C. Heat-denatured (100° C) sheared salmon DNA was added to GEAhyb hybridization solution (provided by the kit). The pre-wetted GEArray membrane was placed into the hybridization bottle, and the membrane was pre-hybridized with the above solution for two hours at 60° C with continuous agitation of 5 to 10 RPM. After this was completed, the membrane was hybridized overnight with the above solution from step (c) and denatured cDNA probe. Overnight incubation was performed at 60° C with continuous agitation at 5 to 10 RPM. The membrane then went through several washes.

(d) Chemiluminescence: GEArrays were then prepared for chemiluminescent detection. First, the arrays were incubated with alkaline phosphatase conjugated streptavidin (AP) for 10 min at 5 to 10 RPM. The GEArrays were then washed multiple times and incubated with 1.0 ml CDP-Star chemiluminescent substrate for 2 to 5 minutes. The wet membrane was immediately wrapped in a hybridization bag and exposed to x-ray film with an intensifying screen.

(e) Chemiluminescent Detection: Optimal exposure time depends on parameters independent of the array itself. In particular, the visualization of high abundance message required shorter exposures and lower abundance messages required longer exposures. Thus, the

membranes were exposed to x-ray film for varying lengths of time. Length of exposure for these experiments was less than 15 seconds to obtain images with maximal signal intensity and minimal background noise. A scanner was used to convert the x-ray image into a gray-scale TIFF file (the membrane is divided into 8 columns and 14 rows with each cell of the grid representing a tetra-spot). The TIFF image was converted into numerical data using the software program Scanalyze (Eisen Software).

(vi) Data and statistical analysis:

Raw data obtained from Scanalyze were further analyzed using the GE Array Analyzer. The GEArrayAnalyzer software, was used to match the raw data to the gene list of the specific array. Plasmid DNA (PUC18) negative controls was used as a reference for background substitution. This manipulation removes the background from the experimental readings by giving a general idea of the lowest amount of exposure possible for signals taken from a particular array and then subtracting this amount from the raw experimental results. GAPDH was used as the positive control for normalization. This normalization method was intended to remove the differences in amount of exposure between experiments, providing a baseline so different experiments were comparable to one another. This was necessary for comparison of different arrays (i.e., such as comparison between two different experimental conditions), since the overall signal of different arrays can fluctuate significantly. The relative abundance of a particular transcript was estimated by comparing its signal intensity to that of the positive controls.

The genes that were assessed on the array are listed in Tables 3, 4, and 5.

(IV) Results

Genes were considered significantly modulated in expression when there was at least a 2-fold difference in expression between cells incubated with *P. gingivalis* extracts and controls at day 4. Furthermore a visual trend in the expression pattern was also observed. Table 6 shows genes that were found to have a significant downregulation of expression in response to extracts from *Porphyromonas gingivalis.* Table 8 shows genes that were found to have a significant upregulation of expression in response to extracts from *Porphyromonas gingivalis.* Genes that were not significantly modulated in expression are listed in Table 7.

An overall low expression of alkaline phosphatase was seen in both control groups at day 0 and at day 4 with a signal intensity of 1 to 2% of maximal expression. An increase in ALP expression was seen in the 1μg/ml and 10μg/ml groups with respective 22.7 and 17.6 fold increases over control culture at day 4. (Figure 1)

Among the downregulated genes, many collagen mRNA subunits were significantly affected by *P. gingivalis* extracts. The most significant downregulation occurred in Type II collagen α1 with 99% decrease in expression as compared to control cultures at day 4 at the 1μg concentration of *P. gingivalis.* (Figure 2) Higher concentrations of *P. gingivalis* (10μg and 100μg) also resulted in a significant mRNA downregulation of type II collagen α1 at 68% and 60% respectively. Collagen, type III α1 had a similar pattern with a decrease of 97.4% (1μg/ml), 70% (10μg/ml), and 65.5% (100μg/ml) in mRNA expression. (Figure 3) Collagen type XI α1 had a respective mRNA downregulation of 54.7% (1μg/ml), 102% (10μg/ml), and 80.9% (100μg/ml). (Figure 4) Collagen type XV α1 gene expression had a respective downregulation of 54.7% (1μg/ml), 102% (10μg/ml), and 80.9% (100μg/ml). (Figure 5) Other collagen subunits such as type IX collagen α2, Type XI collagen α1, Type XIV collagen α1, and Type XVI

collagen α1 showed a definite trend for mRNA downregulation, but not to the degree of the previously mentioned collagen subunits.

Collagen type I mRNA expression was slightly affected by *P. gingivalis* extracts. Control groups at day 0 and day 4 showed a signal 5 to 9% of maximal intensity. Addition of extracts from *P. gingivalis* caused the downregulation of collagen type I expression by 64.9% (1μg/ml), 16.5% (10μg/ml), and 35.7% (100μg/ml). (Figure 6)

Bone morphogenetic proteins, which are important in the cell-signaling cascade of osteogenesis and osteoblast differentiation also demonstrated decreases in expression when exposed to *P. gingivalis* extracts. BMP-2 gene expression was inhibited at day 4 with a 123% decrease from the control at the10 μg/ml concentration. (Figure 7) Overall expression of BMP-2 was low for the control at day 4 with a signal 25% of maximal intensity. BMP-3 showed a similar trend in that the greatest decrease in mRNA expression (110%) was seen on day 4 at the 10μg/ml concentration. (Figure 8)

The Smad group of genes (signaling mothers against decapentaplegic) which are necessary for intracellular signaling via external receptors, showed a significant mRNA downregulation. Most notable was Smad1, which showed 113% decrease in expression at day 4 for the 1μg/ml concentration. (Figure 9) The 10μg/ml and 100μg/ml concentrations also showed mRNA downregulation by 69% and 67% respectively. A downregulation in gene expression was also observed for Smad2, Smad3 and Smad7 genes, although to a lesser extent.

Matrix metalloproteinase expression was generally not affected by *P. gingivalis* extracts. MMP-9 and MMP-10 appeared to have constitutively high expression regardless of the concentration of extracts from *P. gingivalis* while MMP-8 had consistently low expression. However, MMP-2 demonstrated a somewhat dose-dependent inhibitory response to *P. gingivalis*

extracts. Addition of extracts from *P. gingivalis* caused a downregulation of MMP-2 expression by 10.6% (1μg/ml), 52.8% (10μg/ml), and 58.9% (100μg/ml). (Figure 10)

The TGF-associated genes were downregulated in response to *P. gingivalis* extracts. TGF-β 2 demonstrated a trend for increased in expression in the control group with a 49% increase from day 0 to day 4. However, the groups with extracts from *P. gingivalis* showed a downward trend with a 126.1% (1μg/ml), 82.5% (10μg/ml), and 20.3% (100μg/ml) change from control. (Figure 11) Expression of TGF-β 3 in the control groups was consistently high with control expression at 95% and 96% of maximal signal intensity for day 0 and day 4. Extracts of *P. gingivalis* showed a maximal decrease in TGF-β 3 expression of 29% at the 10μg/ml concentration. (Figure 12) A similar trend was seen for TGF-β receptor with high expression in both the day 0 and day 4 controls with signal intensity of 90% and 91% for day 0 and day 4. Maximal decrease in TGF-β receptor expression was seen in the 10ug/ml concentration with a 53.6% decrease. (Figure 13)

Insulin-like growth factor (IGF)-related gene mRNA was significantly downregulated in response to extracts from *P. gingivalis*. IGF-1 had relatively high mRNA expression with 60% and 76% of maximal signal intensity for day 0 and day 4 controls respectively. A 94.6% decrease in IGF-1 expression was seen with 1 μg/ml concentration of extracts from *P. gingivalis*. Higher concentration of extracts showed a similar downregulation of expression, but not to the degree of the 1μg concentration. (Figure 14) IGF-2 expression for the control cultures at day 0 and day 4 was not as high as IGF-1, with expression of 18% and 27% of maximal intensity. Addition of extracts from *P. gingivalis* elicited a strong downregulation in expression of 145.9% (1μg/ml), 101.5% (10μg/ml), and 34.1% (100μg/ml) change from control cultures. (Figure 15) Insulin-like growth factor 1 and 2 both show a similar trend in that the maximal inhibition of IGF expression

by *P. gingivalis* extracts occurs with the 1μg/ml concentration (decrease of 94.6% and 145.9% respectively). The insulin-like growth factor receptor followed the same inhibition trend with maximal mRNA downregulation of 157.6% at the 1μg/ml concentration on day 4. (Figure 16)

The expression of non-collagenous extracellular proteins involved in bone matrix formation that were evaluated included osteopontin and osteocalcin. Osteocalcin showed a significant increase in expression with a 113% increase from day 0 control to day 4 control. This upregulation trend of osteocalcin was significantly downregulated by the addition of extracts from *P. gingivalis*. The day 4 control for osteocalcin did not differ from the 1 μg/ml *P. gingivalis* extracts at day 4, however a dose-dependent response was seen at higher concentrations with a 44% reduction for the 10μg/ml concentration and 51.5% reduction for the 100μg/ml concentration. (Figure 17) Osteopontin showed a significant increase in expression in the controls with a 160% increase from day 0 to day 4. This upregulation trend of osteopontin gene expression was significantly downregulated by the addition of extracts from *P. gingivalis*. Extracts from *P. gingivalis* at concentrations of 1 μg/ml decreased expression by 95.2%, 81.3% (10μg/ml) and 98% (100μg/ml). (Figure 18)

(V) Discussion

Periodontopathogenic bacteria and their products have been previously shown to have a direct effect on periodontal cells such as fibroblasts, macrophages, and epithelium. The complex inflammatory response as a result of exposure of these cells to bacteria or bacterial products induces local soft tissue destruction and resorption of alveolar bone. Periodontopathic bacteria and their products are also thought to have direct influences on osteoblasts, which may inhibit cell differentiation and bone formation. Few studies have investigated the direct effect of bacterial products on osteoblasts. In this study, we examined the effects of extracts from a known periodontopathogenic bacterium, *Porphyromonas gingivalis*, on an osteoblast cell line (hFOB 1.19) *in vitro*. Our results indicate that soluble extracts from *P. gingivalis* inhibit the expression of genes related to matrix production and synthesis of cytokines, growth factors, and internal signaling molecules in cultured osteoblast-like cells (hFOB 1.19).

In this study, the growth media used for the hFOB cells included supplements that promote bone formation, giving us the highest potential for detecting changes in the expression of genes related to bone formation in osteoblasts. For example, the media was supplemented with ß-glycerophosphate that is known to promote mineralization in chick limb bud mesenchymal cells, human and rat marrow cells, and human odontoblastic cells. (Camps et al., 2002)[11] In addition, it has been shown that dexamethasone has the potential to induce cells to proliferate, undergo chemotaxis, and produce type I collagen. (Brenner et al., 2001)[9] (Tennenbaum et al., 1985)[54] Ascorbic acid was also added to the growth media because it is an essential cofactor for lysyl hydroxylase and prolyl hydroxylase, enzymes essential for collagen biosynthesis. Ascorbic acid plays an important role in the formation of collagen in the periodontal ligament and has been shown to stimulate differentiation of murine osteoclasts. (Argab et al., 1998)[3]

(i) Effects on Alkaline Phosphatase:

The inhibitory effects of *P. gingivalis* extracts on osteoblast cell lines have been previously demonstrated. (Kadono et al., 1999)[27] (Loomer et al., 1995)[30] (Meghji et al., 1992)[36] Kadono and co-workers (1999)[27] demonstrated that fetal rat calvaria cells were significantly inhibited by the addition of *P. gingivalis* LPS in the culture media. Alkaline phosphatase is commonly used as a marker enzyme of osteoblast-cell differentiation. Alkaline phosphatase activity was inhibited after 7 days of incubation to approximately 60% of control activity. A similar decrease in alkaline phosphatase activity was reported by Loomer and coworkers (1998)[33] when they exposed various fractions of *P. gingivalis* extracts to chick periosteal calvarial tissues. However, this effect was only noticed when the *P. gingivalis* extracts were added to the culture medium in the differentiation stage (0 to 2 days). If extracts were added after osteoblast differentiation had already occurred, (day 2) no differences in alkaline phosphatase levels were observed. Our results consistently show low signal intensity of alkaline phosphatase mRNA expression in the control groups at day 0 and day 4. An increase in the expression by up to 22 fold was seen in cultures with extracts from *P. gingivalis*. This result is in contrast to what was hypothesized since the hFOB cells express markers that are usually consistent with a well-differentiated osteoblast. A possible explanation for this expression pattern could be due to the relatively short incubation period. Kadono and coworkers (1999)[27] showed a low level of alkaline phosphatase activity in all groups incubated less than 7 days.

(ii) Effects on Collagen:

In addition to the effects on alkaline phosphatase, collagen synthesis has also been shown to be affected by the addition of *P. gingivalis* extracts on osteoblast cell lines. Meghji and co-

workers (1992)[36] demonstrated a 31.5% reduction of collagen synthesis by measuring the incorporation of tritiated proline in collagen. This same method of measuring collagen synthesis was used by Loomer and co-workers (1998)[30] and they found similar patterns of inhibition. In this study, we demonstrated that there was a pronounced downregulation of collagen type II, III and XI when cells were exposed to extracts from *P. gingivalis*. Other collagen subunits such as type IX collagen α2, Type XI collagen α1, Type XIV collagen α1, Type XV collagen α1, and Type XVI collagen α1 showed a trend for downregulation, but not to the extent of the previously mentioned collagen subunits. Interestingly, there was a low level expression of collagen type I the predominant collagen of bone and skin, in both the experimental and control groups. This was probably due to the relatively short incubation period of our cell cultures. In our experiments, the type I collagen mRNA synthesis was slightly downregulated by the extracts from *P. gingivalis*.

(iii) Effects on TGF-β Family Growth Factors and Related Genes:

The transforming growth factors-β superfamily, which includes TGF-β, activins, inhibins and bone morphogenetic proteins (BMP), are structurally related secreted cytokines. Many cellular functions such as proliferation, apoptosis, and migration have been associated with TGF-β activity. (Itoh et al., 2000)[43]

BMPs are members of the TGF-β superfamily of polypeptides and are associated with the differentiation and maturation of osteoblast cells from a mesenchymal cell lineage. They play pivotal roles during early embryonic development and tissue morphogenesis. They can also induce endochondral ossification and chondrogenesis. (Canalis et al., 2003)[12] Their ability to induce differentiation and maturation involves a complex intracellular signaling pathway that

involves the mothers against decapentaplegic (Smad) family of proteins. (Bonewald et al., 1992)[6] Kalajzic and co-workers (2005)[28] used mouse calvaria osteoblast cells. This heterogeneous population was then differentiated between preosteoblastic and mature osteoblast cell lines by fluorescent antibody stains to surface markers of cell differentiation. Microarray studies of the gene expression patterns revealed that the transition to mature osteoblasts involve a 6-fold increase in BMP-8 production and a 2-fold increase in BMP-2 production. Other genes such as BMP-4, BMP-7, and BMP receptor were constitutively expressed. Our results demonstrated a similar upregulation of BMP-2 expression as seen in the controls when comparing day 0 to day 4. However, the addition of *P. gingivalis* extracts to the media significantly reduced the BMP-2 expression to almost undetectable levels at day 4. Interestingly, there was also a reduction in the expression of BMP-3 when exposed to *P. gingivalis* extracts. The inhibitory role of BMP-3 is an exception to the stimulatory roles played by other BMPs in osteoblast differentiation and function. BMP-3 opposes the osteogenic effect on BMP-2 in stromal cells and BMP-3 null mice show an increase in bone mineralization and density. (Canalis et al., 2003)[12] In this study, the expression of the remaining BMPs (1, 4, 5, 6, 7, 8) did not show significant changes from those of the control cultures.

Although TGF- β peptides use the same intracellular pathway (Smad), they have a different biological activity. TFG- β has been shown to stimulate preosteoblastic cell replication, osteoblastic collagen synthesis (Hock et al., 1990)[23], bone matrix apposition and alkaline phosphatase activity. (Bonewald et al., 1992)[6] However, TGF-β has also been shown to oppose the effects of BMPs on osteoblastic cell maturation and can induce the differentiation of stromal cells towards the chondrocytic lineage and retard terminal differentiation of osteoblasts. (Bonewald et al., 1990)[7] Genes involved in the production of TGF-β such as TGF-β 2 and 3 as

well as the TGF receptor showed a downregulation of expression in response to extracts from *P. gingivalis*.

(iv) Effects on Intracellular Signaling Molecules

Signals for members of the TGF-β superfamily are mediated by Smad proteins. Eight mammalian Smad proteins have been identified and further subclassified into subgroups based upon structure and function. The first subgroup, the R-Smads or pathway-restricted Smads directly interact with the serine/threonine kinase receptor on the cell surface. This subgroup contains Smad1, Smad2, Smad3 Smad5, and Smad8. The interaction with a R-Smad and a receptor bound to its respective molecule, activates the R-Smad via phosphorylation. R-Smads are specific in that they are only activated by one member of the TGF superfamily. Smad1, Smad5, and Smad8 are activated by BMP molecules whereas Smad2 and Smad3 are activated by TGF-ß/activin signals. (Itoh et al., 2000)[26] The second subgroup is the C-Smad or common-mediator Smad. Smad4 is a C-Smad and has been the only one identified in mammals. C-Smad functions as a common mediator by binding to activated R- Smads to form a heterodimer complex. Therefore, C-Smad is shared by both BMP and TGF-ß/activin signals. (Miyazono et al., 1999)[38] The heterodimer complex is translocated across the nuclear membrane and regulates the transcription of various target genes. The last subgroup is the I-Smad or inhibitory Smads. Only two I-Smads have been identified in mammalian species, Smad6 and Smad7. They exert their inhibitory effect by stably binding to the serine/threonine kinase receptor and preventing the interaction of this receptor with R- Smads. Smad7 is specific in that it binds to the TGF-ß receptor, but Smad6 can bind to the receptors for TGF-ß, activin, and BMP. (Miyazono et al., 2000)[39] Kalajzic and co-workers (2005)[28] reported no significant changes in any of the Smad

genes during maturation of the osteoblast cell lines. Our results show that the addition of *P. gingivalis*, demonstrated a downregulation of the Smad related genes most notably of Smad1, and with less of an effect on Smad2, Smad3, and Smad7. These results indicate that the addition of extracts from *P. gingivalis* may have a significant interference with the host cell's intracellular signaling.

(v) Effects on Insulin-like Growth Factor and Related Genes

Osteoblast differentiation and osteogenesis is regulated by a number of hormonal and local factors that induce intracellular signaling pathways. Previous studies have shown that deficiencies of the IGF and IGF receptor in osteoblasts were associated with low bone mass and retarded bone cell differentiation. IGF-1 appears to be a principal growth regulator in bone and cartilage and plays a major role in the maintenance of bone mass. (Schwartz et al., 1997)[47] IGF-1 and 2 are mainly synthesized in skeletal tissues including bone fibroblasts and osteoblasts and are upregulated by growth hormones such as estradiol, PGE_2 and inhibited by cortisol. (McCarthy et al., 1990)[35] Both forms of IGF increase preosteoblastic cell replication, have a stimulatory effect on osteoblastic collagen synthesis and bone matrix apposition, and decrease the degradation rate of collagen. (Hock et al., 1988)[22] Kalajzic and coworkers (1999)[28] reported that there were no significant changes in the expression of IGF receptor as cells mature into osteoblasts, but he did find that IGF-1 and IGF-2 expression was reduced approximately 5-fold in the mature cells. In this study, we found that there was a downregulation of both IGF-1 and IGF-2 gene expression when exposed to *P. gingivalis* extracts. The study by Kalajzic and co-workers[28] demonstrated constitutively expressed IGF receptor in the maturing osteoblasts. In contrast, our results show that there was maximal 158% reduction of IGF receptor when exposed

to *P. gingivalis* extracts. The results of this study imply that extracts from *P. gingivalis* significantly downregulate the expression of genes related to insulin-like growth factor.

(vi) Effects on Non-collagenous Bone Proteins:

Other markers of osteoblast cell differentiation and maturation are the non-collagenous proteins inducing osteocalcin and osteopontin. Both of these bone matrix proteins are regulated by calcitropic hormones and growth factors and are closely connected to bone nodule formation. Their expression is considered to characterize mature osteoblasts and to be associated with mineralization of the bone matrix. (Butler et al., 1989)[10] (Lian et al., 1985)[29] Osteocalcin is a specific marker of osteoblastic activity and mineralization of bone matrix because it specifically is synthesized by osteoblasts before being released into the blood or accumulated in mineralized bone. (Weinreb et al., 1990)[56]

Osteopontin is a prominent glycosylated phosphoprotein and is an important bone matrix protein, where it is thought to mediate adhesion of osteoclasts to resorbing bone. However, recent studies have identified an alternative role for osteopontin as a key cytokine regulating tissue repair and inflammation. (Butler et al., 1989)[10] It is involved in a number of physiologic and pathologic events including angiogenesis, apoptosis, inflammation, wound healing and tumor metastasis. (O'Regan et al., 2000)[41] It is mainly synthesized by osteoblasts and osteoclasts and is deposited directly into bone. (Butler et al., 1989)[10] Kadono and co-workers (1999)[27] found that there was a high expression of osteocalcin in the control group while LPS extracts from *P. gingivalis* almost completely inhibited expression toward the later half of the experiment at day 14. A similar high level of osteopontin was found in the control group with moderate suppression of osteopontin mRNA in the experimental group. Our results agree with these

findings in that the hFOB cells exposed to extracts from *P. gingivalis* exhibited downregulation of mRNA for both osteopontin and osteocalcin when concentrations of 10μg/ml or greater of *P. gingivalis* extracts were used. Our results indicate that the extracts from *P. gingivalis* cause a significant downregulation of expression of bone matrix proteins.

(vii) Study limitations and Design:

Direct comparison between our study and previous studies is limited by differences in the experimental protocols used. Some studies used mesenchymal tissue cultures to identify genes associated with bone cell lineage progression and differentiation. (Kadono et al., 1999)[27] (Kalajzic et al., 2005)[28] (Loomer et al., 1995, 1998)[30,33] (Meghji et al., 1992)[36] This approach has inherent difficulties. The major reason for this has to do with the limited number of cells within the culture that become mature osteoblasts. This small proportion of the total population makes data interpretation difficult since it is not certain whether changes in gene expression are occurring in the osteoblast cells or other cells within the population. Furthermore, the growth rates are significantly altered in the immortalized cell lines. Both studies by Kadono et al. (1999)[27] and Meghji et al. (1992)[36] used a heterogeneous population of cells from rat calvaria of which osteoblasts may be in various stages of differentiation and maturation. Our study used a homogeneous immortalized cell line derived from human fetal osteoblasts. This homogeneous population allows us to more accurately determine gene expression changes due to experimental changes in the growth media culture. Previous studies have used an immortalized osteoblast cell line (MG-63) with inherent properties that were not characteristic of native human osteoblasts. (Loomer et al., 2002)[31] The MG-63 human osteosarcoma line does have distinct disadvantages since these cells do not exhibit the complete phenotype of differentiated osteoblasts, have

abnormal growth properties including loss of cell contact inhibition and responses to hormones and cytokines, and have obvious chromosomal defects. The hFOB cell line used in these experiments possesses properties more akin to normal osteoblasts. This cell line exhibits properties such as such as expression of osteoblastic markers, responds to cytokines and hormones, less chromosomal abnormalities, exhibit the matrix synthetic properties of differentiated osteoblasts, and has the ability to form mineralized nodules *in vitro* cultures. These properties of the hFOB cells allow us to make a more direct comparison to our *in vitro* cell cultures to conditions of *in vivo* exposure of tissues to products derived from *P. gingivalis*. Whether this represents the true characteristic of *in vivo* osteoblasts from normal human periodontium to extracts from *P. gingivalis* remains to be elucidated.

Another disadvantage of this study is the lack of parallel run experiments to determine the repeatability and deviations of our results. The cost of the microarrays limited our ability to run triplicate experiments. Consequently, statistical analysis of the data could not be performed. Future studies with the same experimental protocol would provide valuable data whether these results are repeatable.

(VI) Conclusion

1. Sonicated soluble extracts isolated from *P. gingivalis* altered the expression of genes involved in the process of osteogenesis in an *in vitro* culture of hFOB 1.19 osteoblasts. The changes in expression profile were determined using a commercially available gene array (GEArray Q series Human Osteogenesis Gene Array).
2. A decrease in expression was seen in some members of the following family of genes when compared to cells that were not cultured with extracts from *P. gingivalis*:
    a. Collagen production
    b. Bone morphogenetic proteins
    c. Transforming growth factor-β
    d. Intracellular signaling molecules (Smad)
    e. Insulin-like growth factor
    f. Non-collagenous bone proteins (osteopontin and osteocalcin)
3. An increase in expression was seen in alkaline phosphatase compared to cells that were not cultured with extracts from *P. gingivalis*.

(VII) Tables and Figures

Table 1. Virulence factors of *Porphyromonas gingivalis*. (Amano et al., 2003)[1] (Dorn et al., 2002)[15] (Holt et al., 2005)[24] (Ishikawa et al., 1997)[25] (O-Brien-Simpson et al., 2004)[40] (Yilmaz et al., 2006)[58]

Virulence Factor	Description	Effect
Capsule	Polysaccharide heteropolymer that surrounds the outer membrane	• Decreases host ability to activate complement pathway • Inhibits attachment of PDL fibroblasts to root surface • Resistant to host phagocytosis
Lipopolysaccharide	See Table 2	• See Table 2
Fimbriae	Thin, long, single stranded filaments attached to the outer membrane	• Highly antigenic • Essential for adhesion and invasion of host epithelial cells and fibroblasts
Cysteine proteinase (gingipains)	Cysteine endopeptidases	• Proteolytic degradation of host tissues • Upregulation of IL-6 in epithelial cells • Activation of the kallikrein/kinin system • Activation of the blood clotting system • Degradation of fibrinogen and fibrin
Proteinases (general)	Proteinases for bacterial metabolism	• Degradation of type I and IV collagen • Degradation of fibronogens, laminins • Destructions of immunoglobin and components of the complement system • Destruction of host cytokines such as TNF-α, IL-6 • Inhibits generation of oxygen radicals from activated leukocytes
Heat-shock protein (GroEL)	Heat-shock protein	• Highly antigenic • Implicated in eliciting a host cross reactivity and autoimmune response
Hemagglutinins	Surface antigen	• Inhibits hemagglutination • Inhibits binding to epithelial cells • Highly antigenic with a robust humoral response
Hemolysin	Degradation of heme-containing molecules: lactoferrins, transferrins, and hemoglobin	• Degradation of heme containing molecules to provide a high concentration of iron to support bacterial growth
Vesicle formation	Blebs of outer membrane which contains surface antigens and proteases	• Packages virulence factors to induce host response and inflammation

Short-chain fatty acids	Secreted products from *Porphyromonas gingivalis* metabolism	• Inhibitory effect on immune cell
		  ○ Decrease lymphocytic proliferation
		  ○ Decrease cytokine production

Autolysins	Endogenous murein hydrolase capable of degrading cell wall peptidoglycan	• Necessary for cell division • Formation of vesicles • Aids in the release of membrane bound virulence factors

Diagram 1. General architecture of gram-negative bacterial lipopolysaccharide.[55]

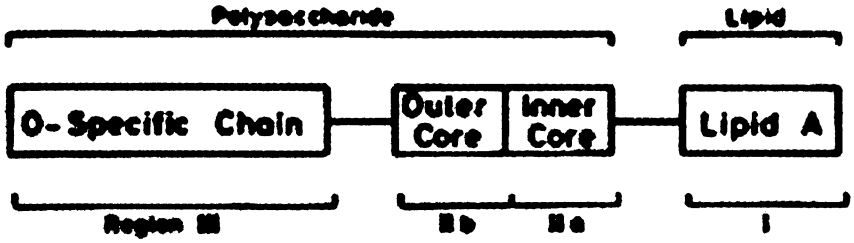

Table 2. Virulence factors specifically associated with LPS components. (Parmanov et al., 2001)[42]

Virulence factor	Description	Effect
O Polysaccharide	• Hydrophilic domain of LPS • Highly variable throughout species	• Mediates adherence to certain tissues, especially epithelial tissues • Resistance to phagocytes • Act as water-solubilizing carriers for toxic Lipid A • Provides protection from damaging reactions with antibody and complement • Antigenic variation guarantees the existence of multiple serotypes of the bacterium
Lipid A	• Hydrophobic domain of LPS • Generally conserved	• Mediates a strong host response o Stimulates production of cytokines, including IL-1, IL-6, IL-8, tumor necrosis factor (TNF) and platelet-activating factor o Activation of the complement cascade. (C3a and C5a) o Activation of the coagulation cascade by Initiating activation of Hageman factor (blood-clotting Factor XII) o B-cell mitogen stimulating the polyclonal differentiation and multiplication of B cells and the secretion of immunoglobulins

Diagram 2. Flow chart of protocol used to assess mRNA expression differences between experimental and control cell cultures.

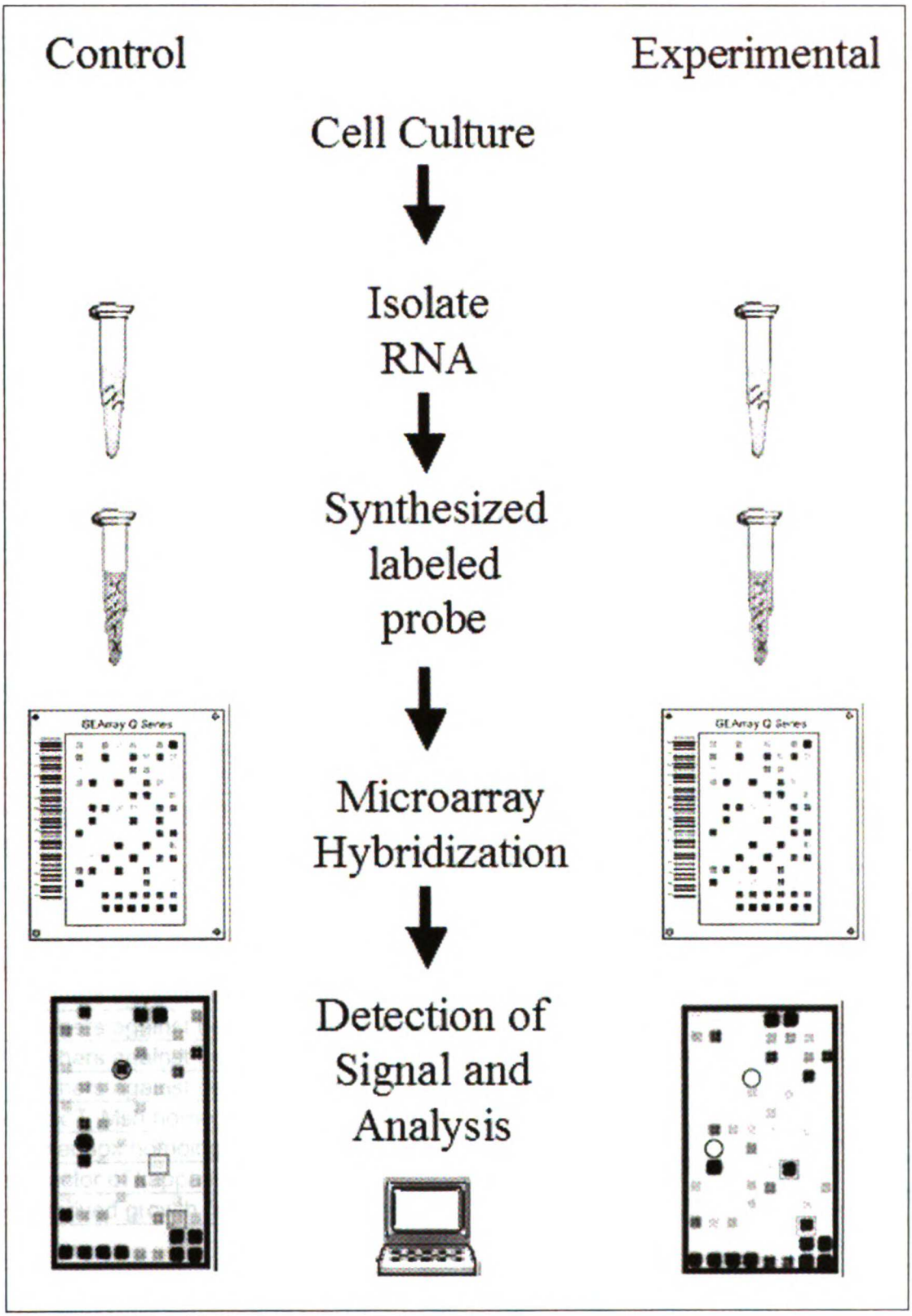

Table 3. GEArray Q series Human Osteogenesis Gene Array. Growth factor genes and associated molecules.

Gene Description	Gene
Bone morphogenetic protein 1	BMP1
Bone morphogenetic protein 2	BMP2
Bone morphogenetic protein 3 (osteogenic)	BMP3
Bone morphogenetic protein 4	BMP4
Bone morphogenetic protein 5	BMP5
Bone morphogenetic protein 6	BMP6
Bone morphogenetic protein 7 (osteogenic protein 1)	BMP7
Bone morphogenetic protein 8 (osteogenic protein 2)	BMP8
Bone morphogenetic protein receptor, type IA	ALK-3
Calcium-sensing receptor (hypocalciuric hypercalcemia 1)	CASR
Colony stimulating factor 2 (granulocyte-macrophage)	GM-CSF
Colony stimulating factor 3 (granulocyte)	G-CSF
Epidermal growth factor (β-urogastrone)	EGF
Epidermal growth factor receptor	EGFR
Fibroblast growth factor 1 (acidic)	FGF1
Fibroblast growth factor 2 (basic)	FGF2
Fibroblast growth factor 3 (murine mammary tumor virus integration site)	FGF3 (int-2)
Fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2)	FGFR1 (FLG)
Fibroblast growth factor receptor 2	FGFR2
Fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism)	FGFR3
Vascular endothelial growth factor receptor (FLT1) mRNA	FLT1
Growth differentiation factor 10	BMP3B
Insulin-like growth factor 1 (somatomedin C)	IGF-1
Insulin-like growth factor 1 receptor	IGF-1R
Insulin-like growth factor 2 (somatomedin A)	IGF-II
MAD (mothers against decapentaplegic, Drosophila) homolog 1	SMAD1
MAD (mothers against decapentaplegic, Drosophila) homolog 2	SMAD2
MAD (mothers against decapentaplegic, Drosophila) homolog 3	SMAD3
Human homozygous deletion target in pancreatic carcinoma (DPC4)	DPC4/SMAD4
MAD (mothers against decapentaplegic, Drosophila) homolog 5	SMAD5
MAD (mothers against decapentaplegic, Drosophila) homolog 6	SMAD6
MAD (mothers against decapentaplegic, Drosophila) homolog 7	SMAD7
MAD (mothers against decapentaplegic, Drosophila) homolog 9	SMAD9
Homeobox 7, Msh homeo box homolog 1 (Drosophila)	Hox7
MSH homeobox homolog 2	MSX2
Nuclear factor of kappa light polypeptide gene enhancer in B-cells	NFkB
Platelet-derived growth factor α polypeptide	PDGFa

Table 3. Continued

Gene Description	Gene
Runt-related transcription factor 2	CBFA1
SRY-box 9	SOX9
Transforming growth factor, β1	TGF β1
Transforming growth factor, β2	TGF β2
Transforming growth factor, β3	TGF β3
Transforming growth factor, β receptor I (activin A receptor type II-like kinase)	TGFBR1 (ALK-5)
Transforming growth factor, β receptor II	TGFbR2
Tumor necrosis factor (TNF superfamily, member 2)	TNFa
Basic helix-loop-helix transcription factor; Homolog of Drosophilia TWIST	TWIST
Vitamin D hormone receptor	VDR
Vascular endothelial growth factor	VEGF
Vascular endothelial growth factor B	VEGF-B
Vascular endothelial growth factor C	VEGF-C

Table 4. GEArray Q series Human Osteogenesis Gene Array. Matrix genes and its associated proteins.

Gene Description	Gene
Alkaline phosphatase, liver/bone/kidney	ALP
Annexin V	Annexin v
Arylsulfatase E	ARSE
Bone gamma-carboxyglutamate (gla) protein	Osteocalcin
Human hPGI mRNA encoding bone small proteoglycan I (biglycan)	Biglycan
CD36 antigen (collagen type I receptor, thrombospondin receptor)	CD36
CD36 antigen-like 1	CD36L1
CD36 antigen-like 2	CD36L2
Type X collagen α1	COL10A1
Type XI collagen α1	COL11A1
Type XII collagen α1	COL12A1
Type XIV collagen α1	COL14A1
Type XV collagen α1	COL15A1
Type XVI collagen α1	COL16A1
Type XVII collagen α1	COL17A1
Type XVIII collagen α1	Endostatin
Type XIX collagen α1	COL19A1
Type I collagen (α)	COL1A1
Type II collagen α1	COL2A1
Type III collagen α1 (Ehlers-Danlos syndrome type IV)	COL3A1
Type IV collagen α3	COL4A3
Type IV collagen α4	COL4A4
Type IV collagen α5	COL4A5
Type V collagen α1	COL5A1
Type VII collagen α1	COL7A1
Type IX collagen α2	COL9A2
Cathepsin K	CTSK
Decorin	Decorin
Fibronectin 1	fibronectin-1
Matrix metalloproteinase 2 (72kD gelatinase, 72kD type IV collagenase)	gelatinase A
Matrix metalloproteinase 8 (neutrophil collagenase)	MMP-8
Matrix metalloproteinase 9 (92kD gelatinase, 92kD type IV collagenase)	gelatinase B
Matrix metalloproteinase 10 (stromelysin 2)	MMP-10
Matrix metalloproteinase 13	collagenase-3
Serine (or cysteine) proteinase inhibitor, clade H, member 1	CBP1
Serine (or cysteine) proteinase inhibitor, clade H, member 2	CBP2
Homo sapiens secreted protein, acidic, cysteine-rich (osteonectin) (SPARC)	SPARC cleavage product
Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I)	OPN

Table 5. GEArray Q series Human Osteogenesis Gene Array. Genes of cell adhesion molecule.

Gene Description	Gene
Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	ICAM-1
Integrin, α1	Integrin a1
Integrin, α2 (CD49B, α 2 subunit of VLA-2 receptor)	Integrin a2/LFA1b
Integrin, α3 (antigen CD49C, α 3 subunit of VLA-3 receptor)	Integrin a3
Integrin, αM (complement component receptor 3, α)	Integrin aM
Integrin, αV (vitronectin receptor, alpha polypeptide, antigen CD51)	Integrin aV
Integrin, β1 (fibronectin receptor, β polypeptide, antigen CD29)	Integrin b1
Vascular cell adhesion molecule 1	VCAM-1

Table 6. Genes found to have a significant downregulation of expression in response to extracts from *Porphyromonas gingivalis*.

	Percent change from control cultures in gene expression at day 4		
Concentration of extracts from *P. gingivalis*	**1μg**	**10μg**	**100μg**
Bone gamma-carboxyglutamate (gla) protein (osteocalcin)	4.30%	-43.95%	-51.42%
Bone morphogenetic protein 2	-92.43%	-122.97%	-82.76%
Bone morphogenetic protein 3 (osteogenic)	-40.39%	-110.16%	-78.35%
Type XI collagen α1	-54.68%	-101.96%	-80.89%
Type XIV collagen α1	-72.25%	-69.40%	-66.19%
Type XV collagen α1	-146.03%	-119.74%	-97.07%
Type XVI collagen α1	-47.75%	-91.78%	-54.61%
Type II collagen α1	-98.98%	-68.60%	-60.33%
Type III, collagen α1	-97.44%	-69.91%	-65.47%
Type IV collagen α3	-165.18%	-124.48%	-81.68%
Type IX collagen α2	-118.09%	-14.36%	-43.43%
Colony stimulating factor 2 (granulocyte-macrophage)	-175.75%	-105.63%	-62.70%
Colony stimulating factor 3 (granulocyte)	-197.03%	-99.46%	-83.45%
Cathepsin K	-153.69%	-126.28%	-97.32%
Fibroblast growth factor 3	-194.50%	-122.40%	-84.52%
Fibroblast growth factor receptor 3	-76.99%	-127.74%	-59.47%
Insulin-like growth factor 1 (somatomedin C)	-94.60%	-69.87%	-53.27%
Insulin-like growth factor 1 receptor	-157.58%	-95.46%	-69.73%
Insulin-like growth factor 2 (somatomedin A)	-145.92%	-101.49%	-34.07%
Integrin, α1	-17.38%	-46.63%	-15.59%
Integrin, α2 (CD49B, alpha 2 subunit of VLA-2 receptor)	-58.00%	-120.57%	-25.32%
Integrin, αV (vitronectin receptor, alpha polypeptide, CD51)	-161.83%	-92.04%	-84.78%
MAD (mothers against decapentaplegic, Drosophila) homolog 1	-113.15%	-69.42%	-67.10%
MAD (mothers against decapentaplegic, Drosophila) homolog 2	-28.63%	-8.28%	-27.96%
MAD (mothers against decapentaplegic, Drosophila) homolog 3	-22.67%	-60.11%	-64.90%
MAD (mothers against decapentaplegic, Drosophila) homolog 7	-133.96%	-37.25%	-63.35%
Matrix metalloproteinase 13	-30.09%	-59.41%	-45.02%
Matrix metalloproteinase 2	-10.59%	-52.79%	-58.82%
Homo sapiens secreted protein, acidic, (osteonectin) (SPARC)	-63.16%	3.51%	3.93%
Secreted phophoprotein 1 (osteopontin, bone sialoprotein 1)	-95.24%	-81.30%	-97.93%
Transforming growth factor, β2	-126.31%	-82.49%	-20.28%
Transforming growth factor, β3	-25.68%	-39.10%	-27.74%
Transforming growth factor, β receptor I	-30.34%	-53.56%	-41.40%
Vascular endothelial growth factor	-147.89%	-104.86%	-38.80%
Vascular endothelial growth factor C	-20.36%	-51.33%	-18.35%
β actin	-37.53%	-71.20%	-39.29%

Table 7. Genes found to have no change in expression in response to extracts from *Porphyromonas gingivalis.*

Genes
Annexin V
Arylsulfatase E
Human hPGI mRNA encoding bone small proteoglycan I (biglycan)
Bone morphogenetic protein 1
Bone morphogenetic protein 5
Bone morphogenetic protein 6
Bone morphogenetic protein 7 (osteogenic protein 1)
CD36 antigen-like 2
Type X collagen α1
Type XII collagen α1
Type XVII collagen α1
Type XIX collagen α1
Type I collagen (α)
Type IV collagen α4
Type IV collagen α5
Epidermal growth factor (β-urogastrone)
Vascular endothelial growth factor receptor (FLT1) mRNA
Fibronectin 1
Growth differentiation factor 10
Integrin, α3 (antigen CD49C, α 3 subunit of VLA-3 receptor)
Integrin, αM (complement component receptor 3, α)
MAD (mothers against decapentaplegic, Drosophila) homolog 5
MAD (mothers against decapentaplegic, Drosophila) homolog 9
Matrix metalloproteinase 10 (stromelysin 2)
Matrix metalloproteinase 8 (neutrophil collagenase)
Matrix metalloproteinase 9 (92kD gelatinase, 92kD type IV collagenase)
Nuclear factor of kappa light polypeptide gene enhancer in B-cells
Platelet-derived growth factor alpha polypeptide
Transforming growth factor, β1
Transforming growth factor, β receptor II
Tumor necrosis factor (TNF superfamily, member 2)
Vascular cell adhesion molecule 1
Vascular endothelial growth factor B

Table 8. Genes found to have an increase in expression in response to extracts from *Porphyromonas gingivalis.*

	Fold increase in gene expression at day 4		
Concentration of extracts from PG	**1 μg/ml**	**10 μg/ml**	**100 μg/ml**
Alkaline phosphatase	22.7	17.6	2.7

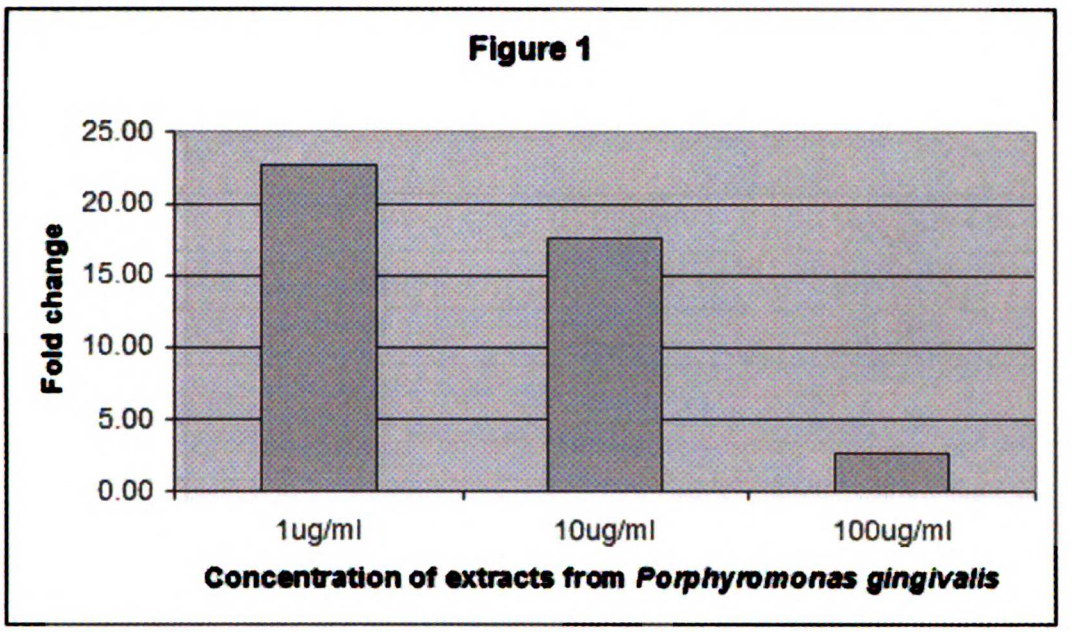

Figure 1. X-fold change in gene expression of alkaline phosphatase at day 4 as a function of extract contraction

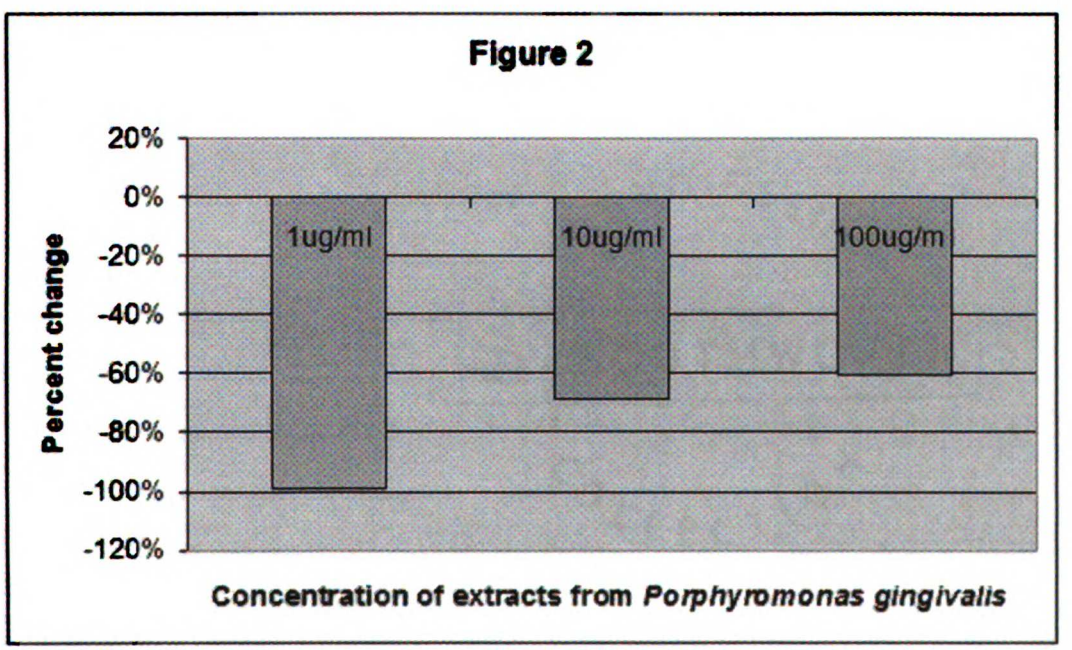

Figure 2. Percent change from control cultures in gene expression of collagen type II α1 at day 4 as a function of extract contraction.

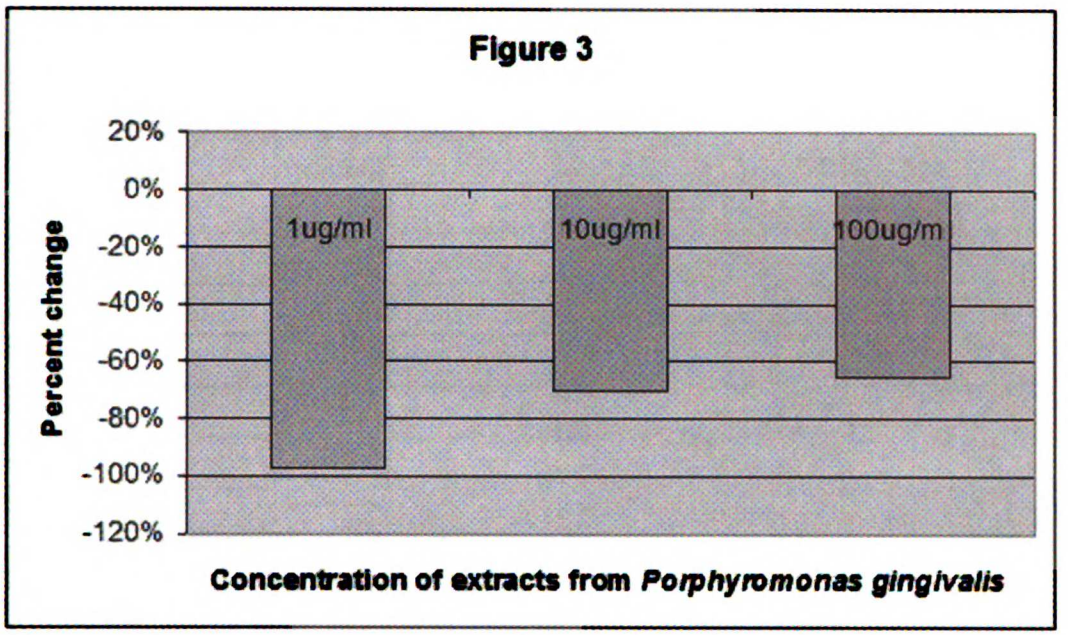

Figure 3. Percent change in gene expression of collagen type III α1 at day 4 as a function of extract contraction.

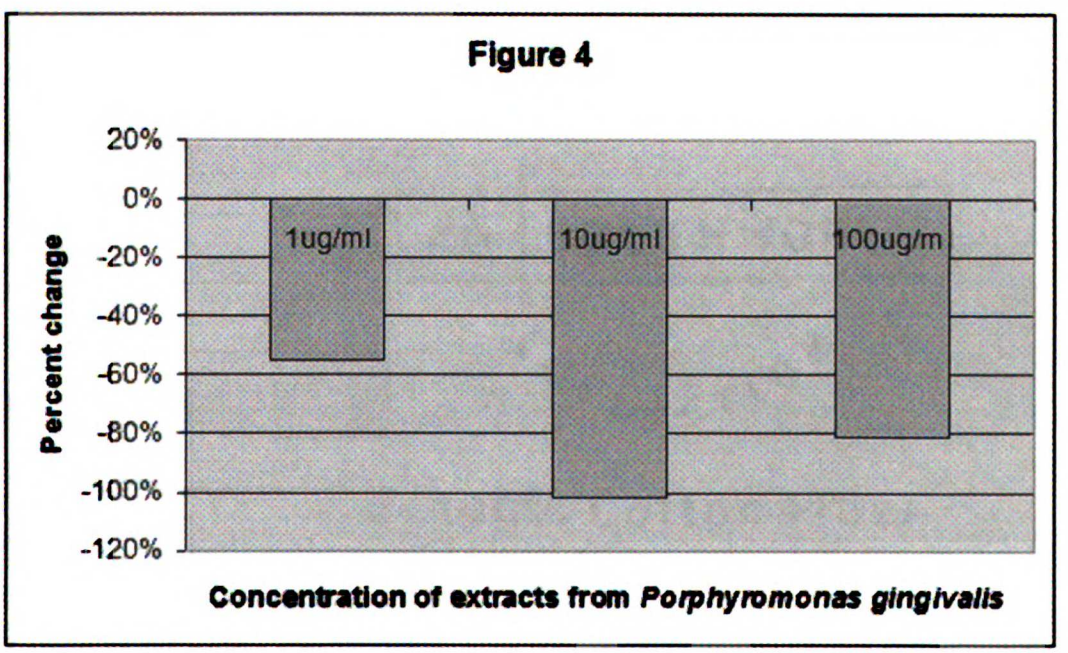

Figure 4. Percent change in gene expression of collagen type XI α1 at day 4 as a function of extract contraction.

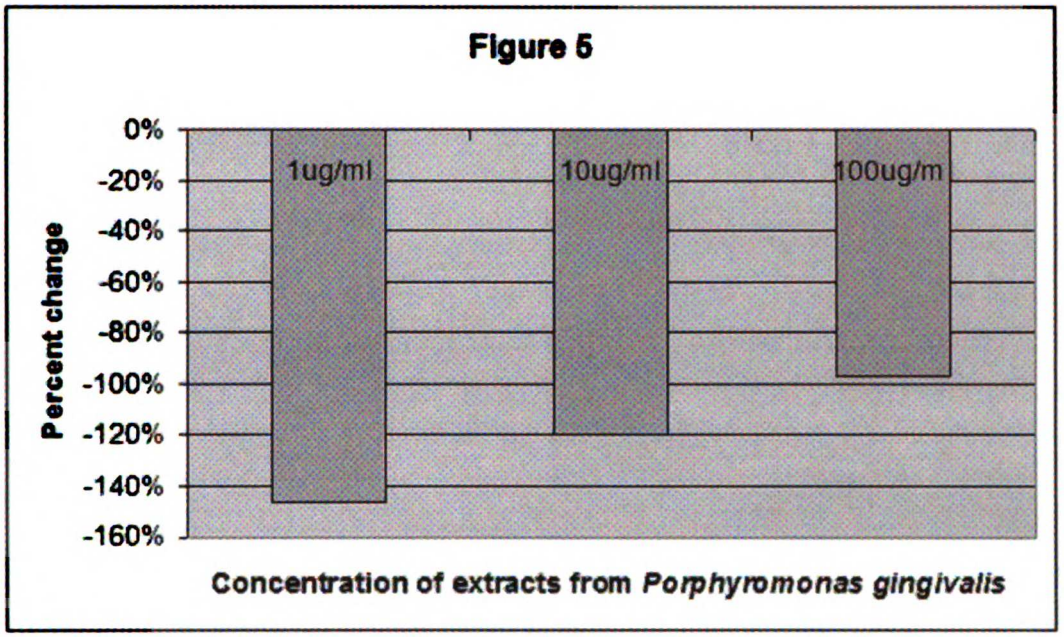

Figure 5. Percent change in gene expression of collagen type XV α1 at day 4 as a function of extract contraction.

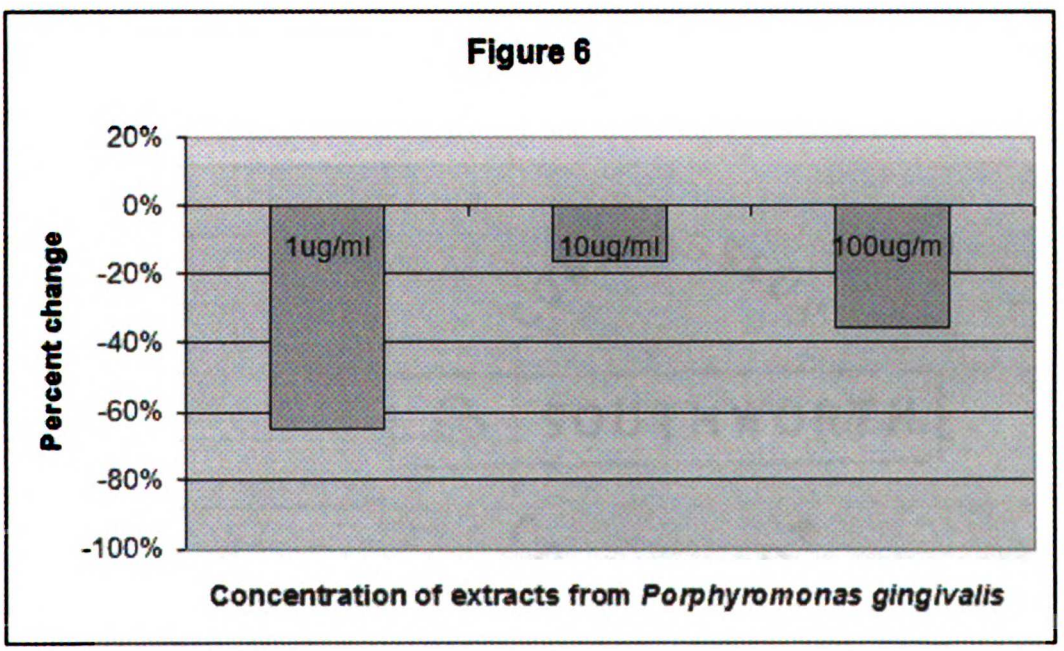

Figure 6. Percent change in gene expression of collagen type I α1 at day 4 as a function of extract contraction.

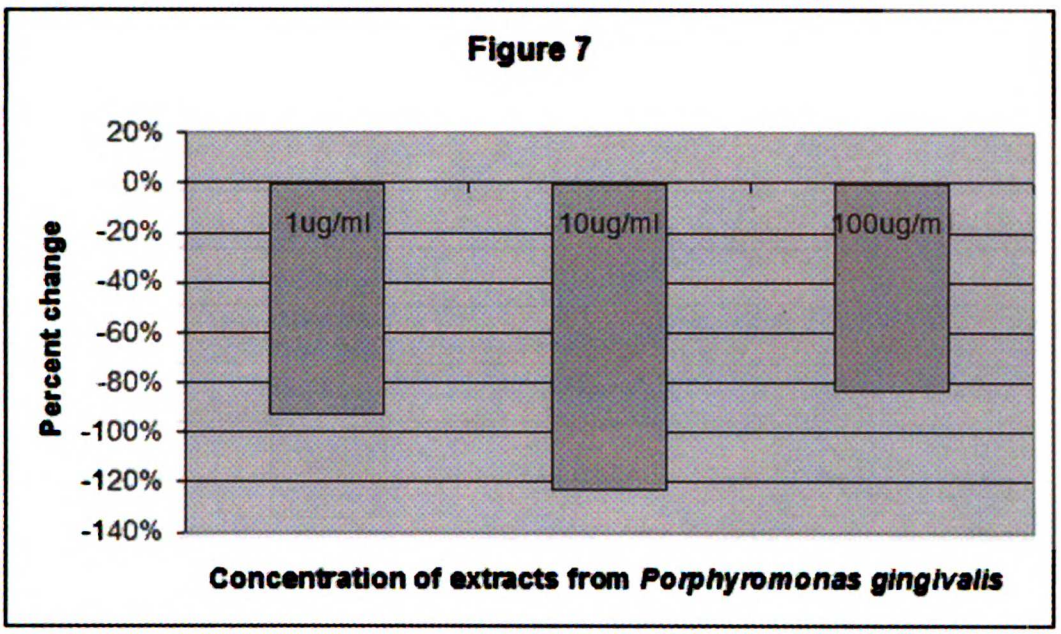

Figure 7. Percent change in gene expression of BMP-2 at day 4 as a function of extract contraction.

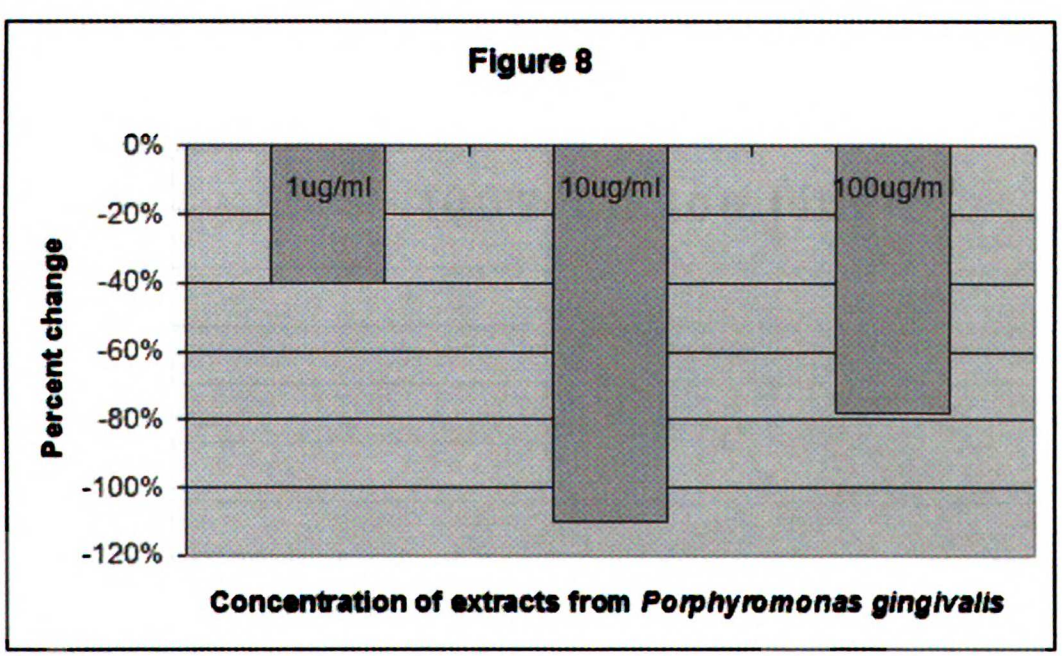

Figure 8. Percent change in gene expression of BMP-3 at day 4 as a function of extract contraction.

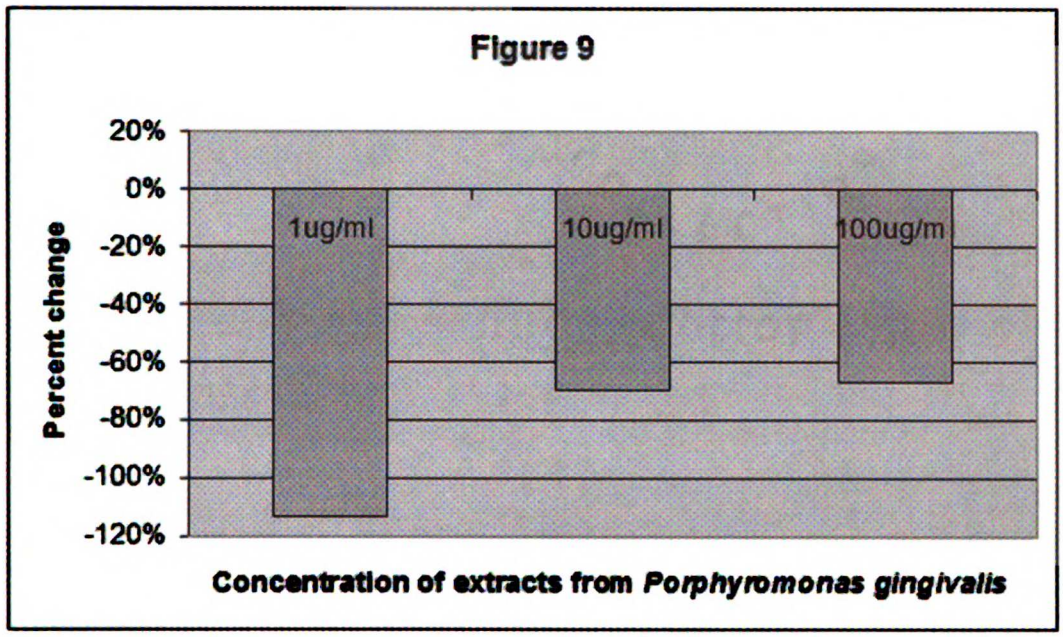

Figure 9. Percent change in gene expression of Smad1 at day 4 as a function of extract contraction.

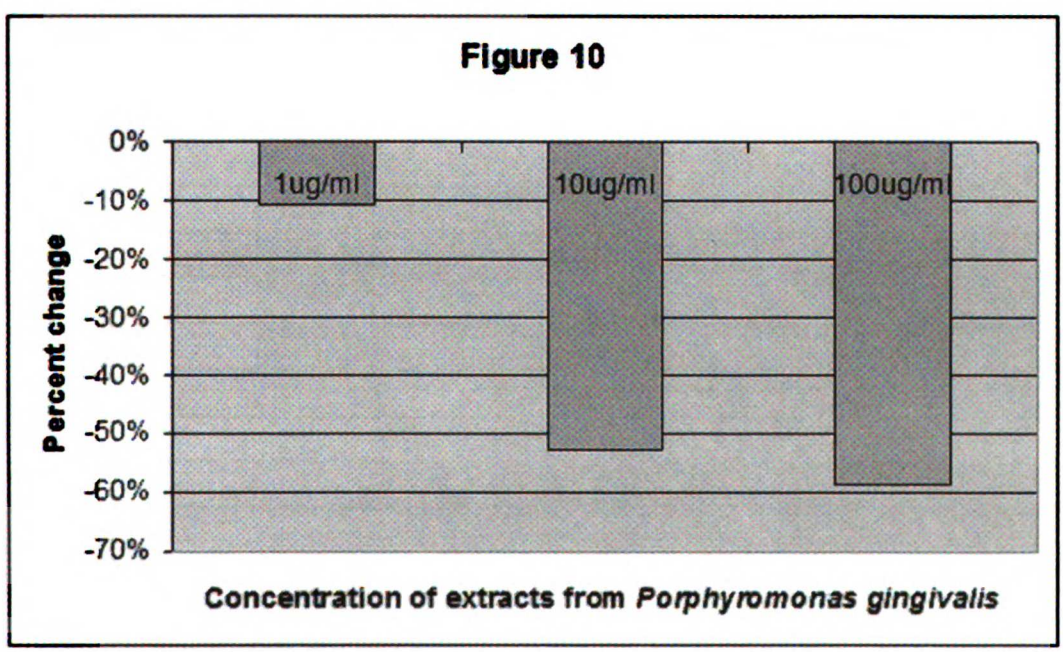

Figure 10. Percent change in gene expression of MMP-2 at day 4 as a function of extract contraction.

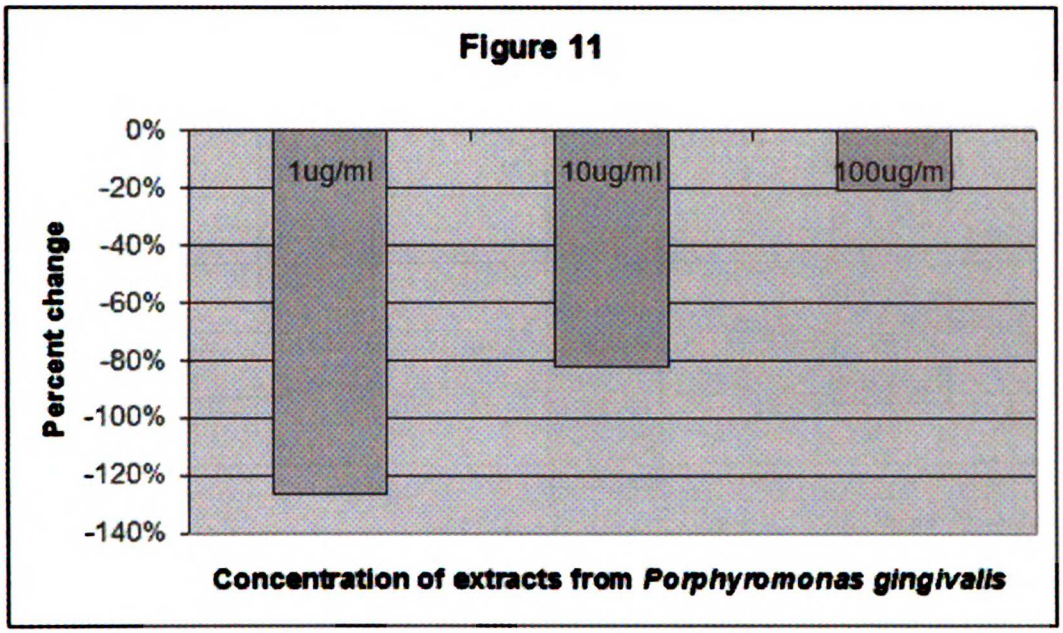

Figure 11. Percent change in gene expression of TFG β2 at day 4 as a function of extract contraction.

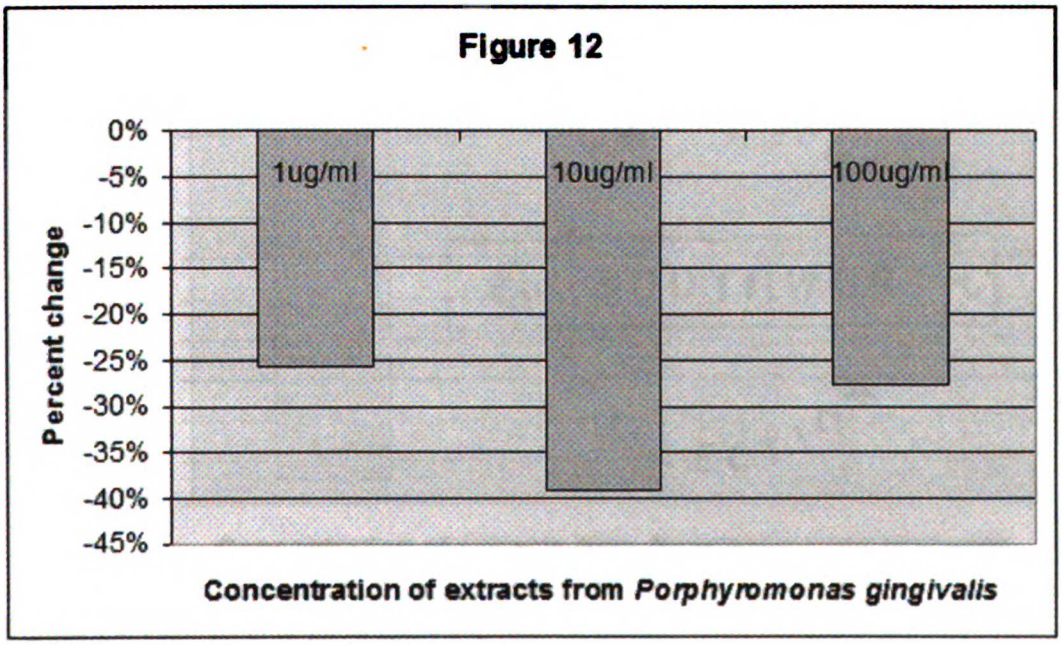

Figure 12. Percent change in gene expression of TFG β3 at day 4 as a function of extract contraction.

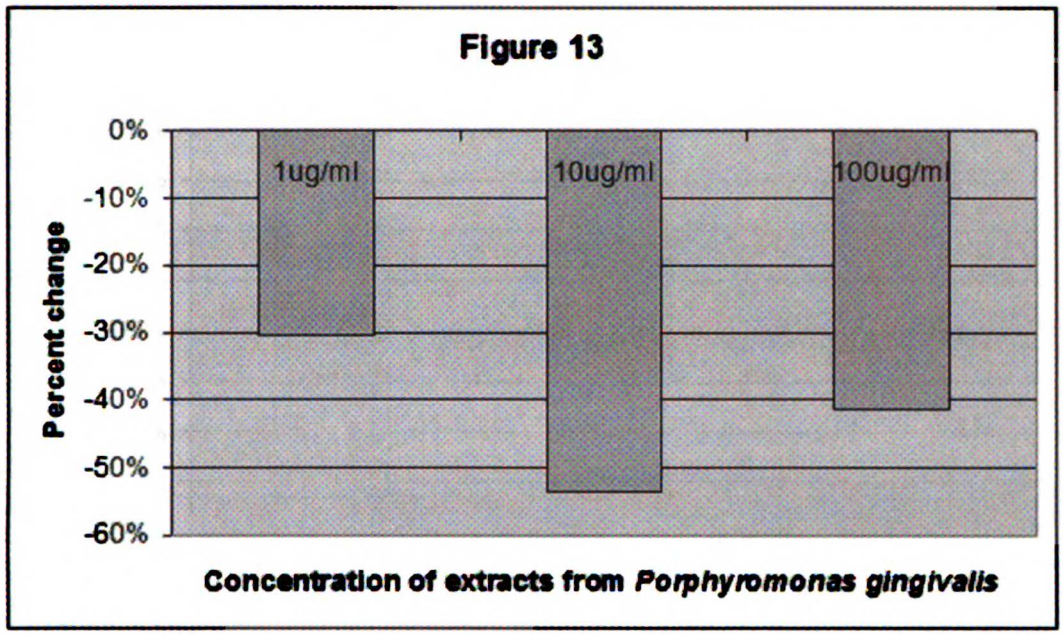

Figure 13. Percent change in gene expression of TFG β receptor 1 at day 4 as a function of extract contraction.

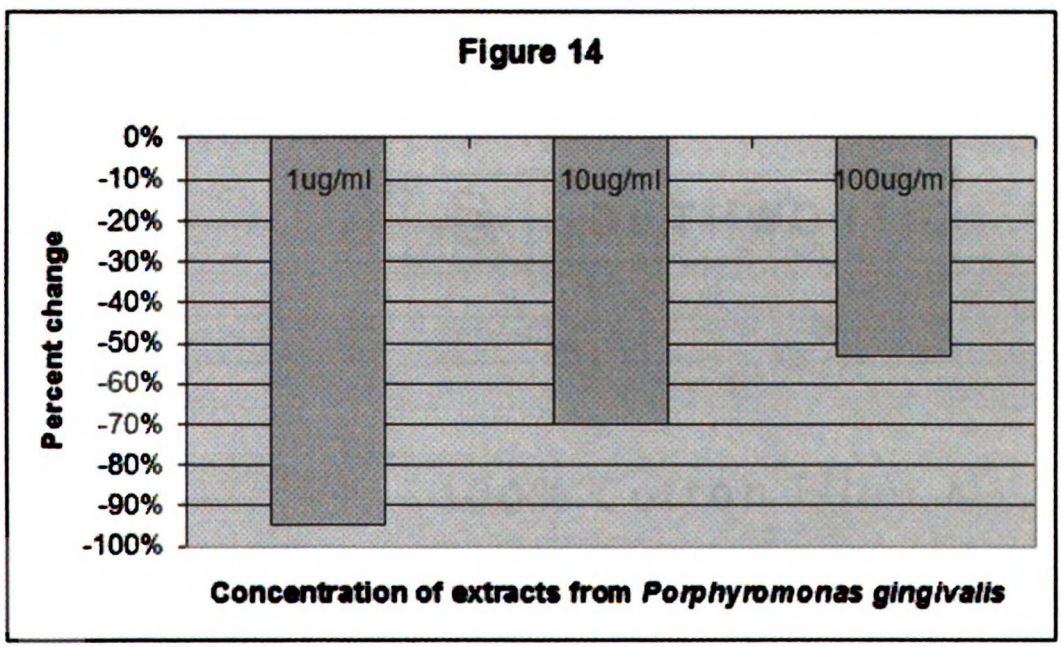

Figure 14. Percent change in gene expression of IGF factor 1 (somatomedin C) at day 4 as a function of extract contraction.

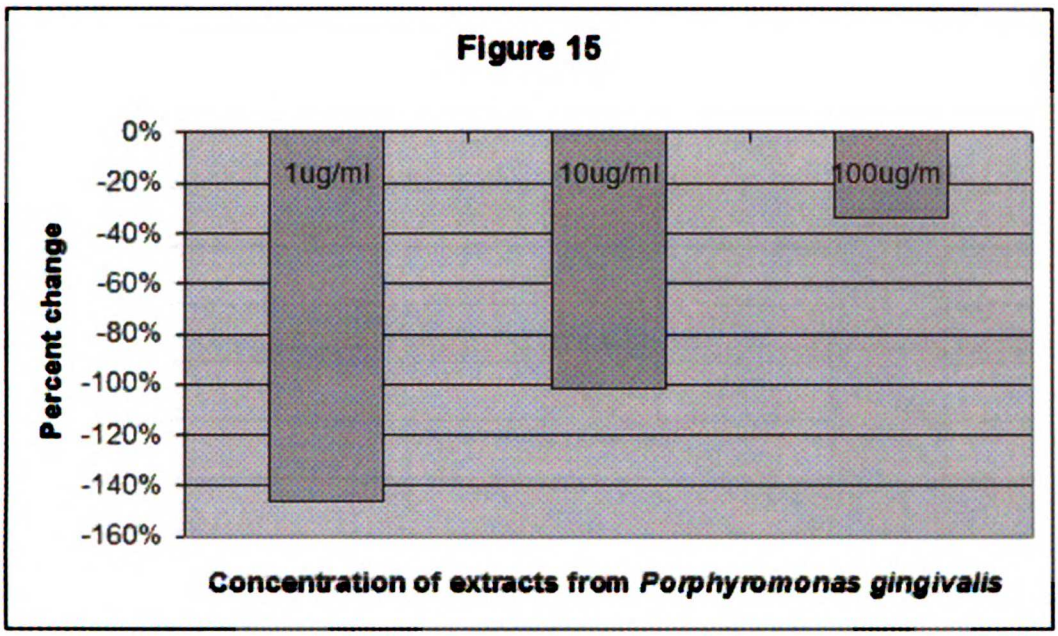

Figure 15. Percent change in gene expression of IGF factor 2 (somatomedin A) at day 4 as a function of extract contraction.

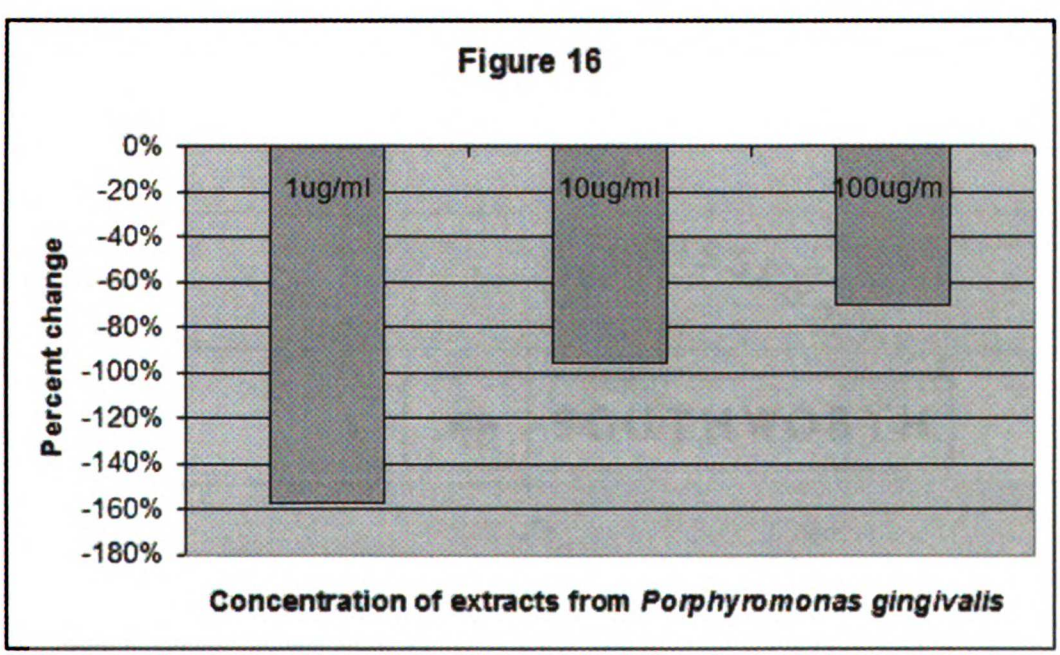

Figure 16. Percent change in gene expression of insulin-like growth factor 1 receptor at day 4 as a function of extract contraction.

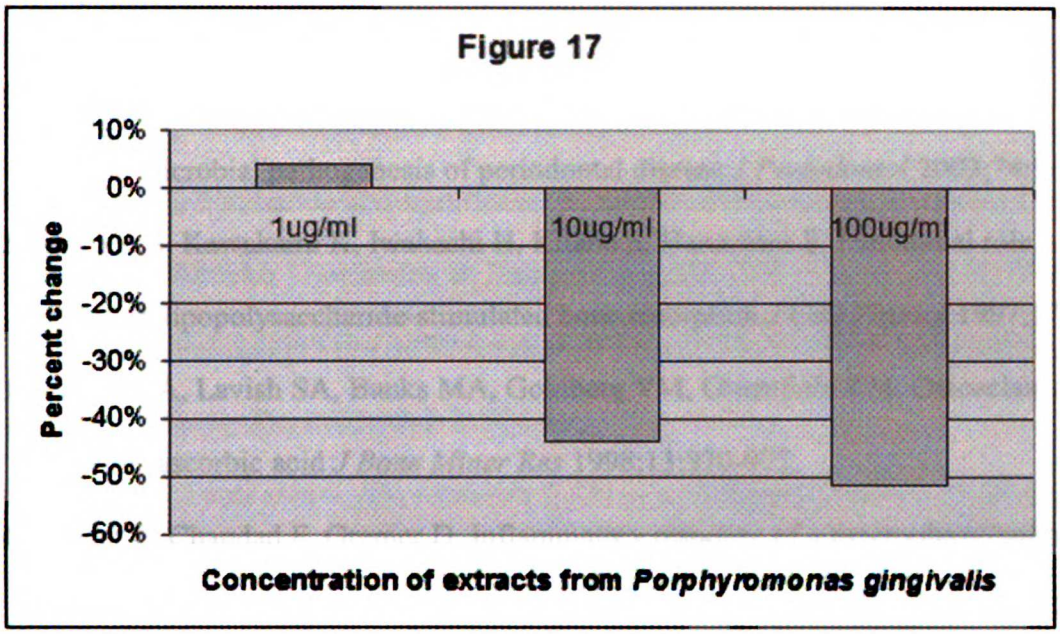

Figure 17. Percent change in gene expression of osteocalcin at day 4 as a function of extract contraction.

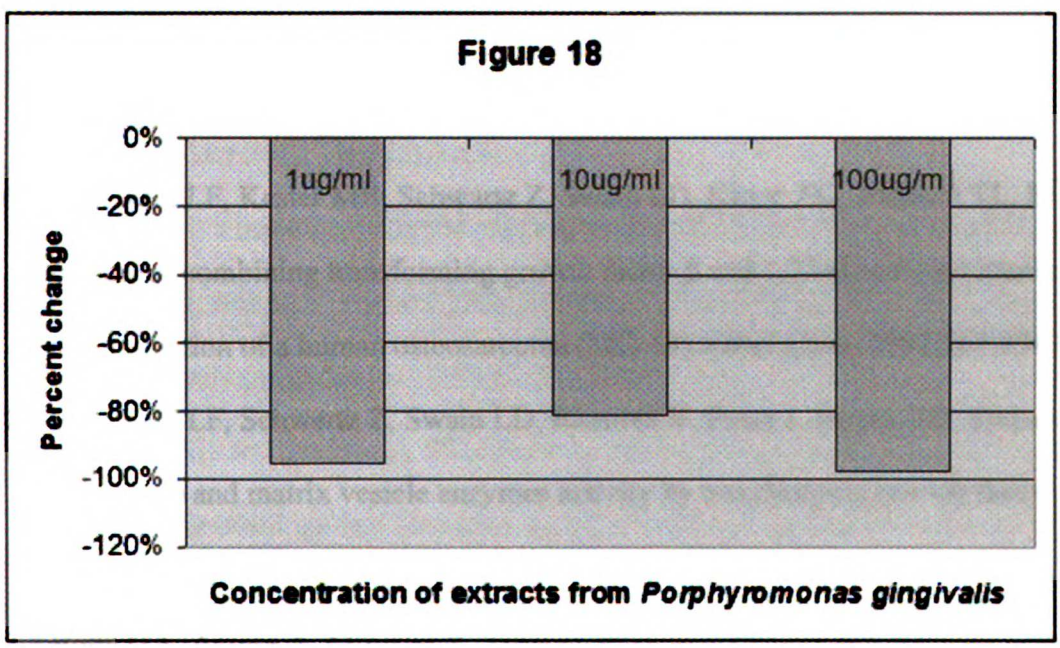

Figure 18. Percent change in gene expression of osteopontin at day 4 as a function of extract contraction.

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